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CONTENTS

SEASONAL CHANGES IN THE OVULATION RESPONSE OF XENOPUS LAEVIS TO METHYL TESTOSTERONE

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In a previous communication [Shapiro, 1936] it was shown that methyl testosterone, in common with certain other steroids, can induce ovulation in *Xenopus laevis*, the South African clawed frog. Zwarenstein [1936] showed previously that progesterone could also induce ovulation in *Xenopus*. His results have been reported more fully [1937].

Xenopus does not ovulate spontaneously under laboratory conditions of captivity. Even at the height of the breeding season, sexual activity disappears as soon as the animals are brought into the laboratory. The animal appears therefore specially suited for the investigation of gonadotrophic substances.

Shapiro and Shapiro [1934] described a seasonal cycle in the gonad ratio, i.e. the mass of the ovaries relative to the body-weight of the animal. This ratio reached a peak during the breeding season, which in South Africa extends approximately from July to September.

The following investigation was therefore undertaken with the object of constructing dose-response curves at various times throughout the year in order to see whether physiological data about seasonal changes in gonadal sensitivity could be obtained in confirmation of the previous evidence, which was entirely gravimetric.

The construction of such curves would also form a useful basis for the investigation of the suitability of *Xenopus* as a test-object for the biological standardization of those steroids which are gonadotrophic in *Xenopus*. The biological standardization of these steroids would be simpler and much more rapid by means of *Xenopus* than by means of any of the current methods employing mammals.

METHODS

Adult female *Xenopus* frogs were obtained fresh from the ponds in July 1937 and in January, April, and August 1938. Two hundred or more animals were obtained in each batch, and were kept in running water for one or two days before being used in an experiment. The animals were not fed.

A stock solution of methyl testosterone in nut oil, 5·0 mg. per ml., was made. Further dilutions were made from this stock solution as required.

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The dose to be injected into each animal was contained in 0·2 ml. The volume of fluid injected was therefore constant throughout the duration of the experiments.

All injections were given intraperitoneally, in the manner described by Shapiro and Zwarenstein [1935]. Immediately after injection each female was placed in a glass container (of capacity 1 pint) half filled with tap-water and provided with a perforated screw top. Usually 2, sometimes 3, dose levels were investigated at the same time. At least 40 animals were injected at each dose level. On the basis of the 1st day's results, new dose levels were tested on the following day, until sufficient data were available to construct a dose-response curve.

The injections were given at 2 p.m. on the day of an experiment. Readings were taken the following morning at 9 a.m. The animals were kept at room temperature.

In the reading of a test all the animals which had oviposited were counted and set aside. The remainder, the apparent negatives, were killed by immersion for about 10 minutes in a mixture of ether and water. They were then dissected to expose the oviducts, which were examined for the presence of eggs within them. The eggs, when present, can be seen easily through the thin wall of the oviduct. The stomach was also inspected as a matter of routine, as occasionally an animal would eat its oviposited eggs. The animals with eggs in their oviducts were counted separately, and are shown in Table I under the heading 'Ovulation', to distinguish them from the animals which had oviposited. The sum of the ovulating and ovipositing animals was taken for the purpose of calculating the response per cent.

RESULTS

The results of the four experiments are shown in Table I.

It is necessary to point out that during the anoestrous months in between the breeding seasons certain animals are injected which appear to be normal. They fail to oviposit. The routine post-mortem examination of the apparent negatives shows that these animals are incapable of ovulating owing to the severely atrophied condition of their ovaries. The number of animals with such completely atrophied ovaries is deducted from the total number of animals injected, before the response per cent. is calculated.

The results in Table I are shown graphically in Fig. 1, the response per cent. being plotted against the dose in micrograms.

DISCUSSION

The characteristic curves obtained are of a type commonly observed in the plotting of biological data. It is clear that the gonadal sensitivity of

the animals shows marked seasonal variations. The curves for July 1937 and August 1938 are very steep. The dose required to produce a 50% response (conveniently referred to as OD. 50) is relatively small, viz. 33·0 µg. in August. These two curves fall well within the breeding season (July to September in South Africa). The two curves for January and for April 1938, which fall in between the breeding seasons, slope very

Table I. *Response of* Xenopus laevis *to methyl testosterone*

Date	Dose in µg.	Oviposition	Ovulation	Apparent negatives	Atrophied ovaries	True negatives i.e. 'apparent negatives' minus 'atrophied ovaries'	Total positives		Per cent. positive
1937	25	2	3	35	Nil	35	5 out of 40		12·5
July	50	23	12	35	Nil	35	35 „	70	50·0
22–9	125	11	17	12	Nil	12	28 „	40	70·0
	250	18	16	6	Nil	6	34 „	40	85·0
	500	27	9	4	Nil	4	36 „	40	90·0
1938	50	3	Nil	37	7	30	3 out of 33		9·0
January	150	10	11	52	9	43	21 „	64	33·0
4–6	300	14	4	22	4	18	18 „	36	50·0
	400	11	13	16	2	4	24 „	38	63·0
	800	23	7	9	4	5	30 „	35	86·0
1938	50	Nil	Nil	40	Nil	40	0 out of 40		0·0
April	150	5	6	29	Nil	29	11 „	40	28·0
20–3	300	13	5	22	Nil	22	18 „	40	45·0
	500	19	4	17	2	15	23 „	38	60·0
1938	25	12	14	34	Nil	34	26 out of 60		44·0
August	50	14	12	14	Nil	14	26 „	40	65·0
8–10	150	25	9	6	Nil	6	34 „	40	85·0

much more gradually to a maximum. At this time of the year large differences in dosage produce relatively small differences in response. OD. 50 is now approximately 360 µg. [April 1938]. The gonadal sensitivity is therefore decreased by about elevenfold in between the breeding seasons.

It is of interest to note that a closely corresponding change occurs in the minimum effective dose, i.e. the least dose capable of producing any response at all (M.E.D.). In August 1938 the M.E.D. was 5·0 µg., whereas in April 1938 it was 55·0 µg. The M.E.D. therefore increased elevenfold when the sensitivity of the ovaries was diminished elevenfold.

In performing the experiments, attempts were made to inject the animals at dose levels which would give responses in the neighbourhood of 50%. With two such points, e.g. in the August 1938 data, 44% for 25 µg., and 65% for 50 µg., the logarithm of the dose in micrograms can be plotted against the response per cent. when a straight line is obtained. This has been done for all four experiments in Fig. 2.
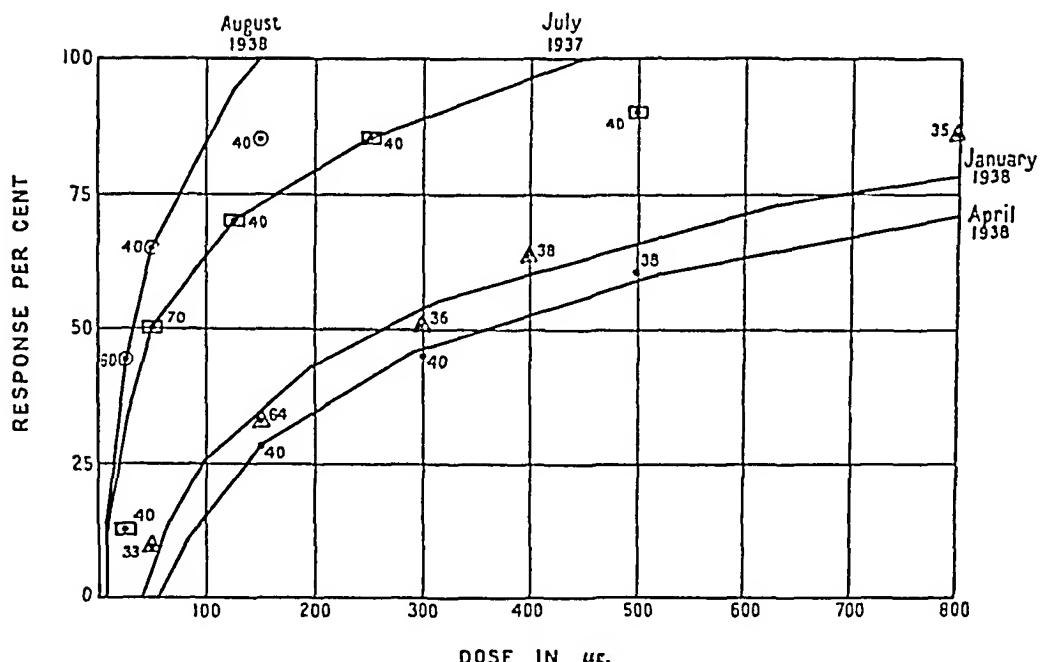
The number of animals injected for each experimentally determined point is indicated by a number beside that point.

FIG. 1. Seasonal variations in the response of *Xenopus lacvis* to methyl testosterone.
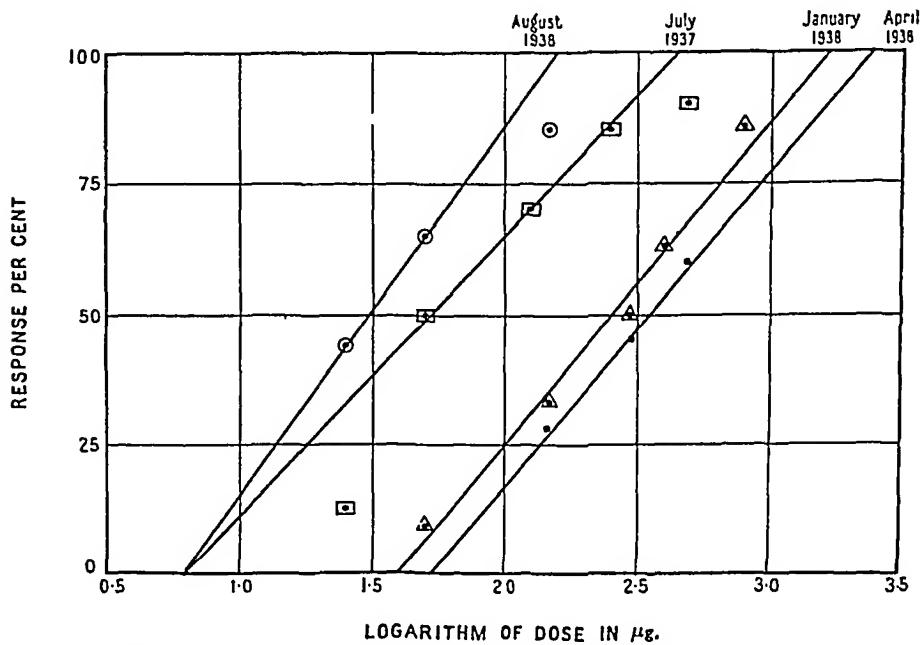
FIG. 2. Logarithm of dose in micrograms against response per cent.

The results for August 1938 fall on a much more steeply sloping line than do those for July in the previous year. It is therefore possible that the peak curve may be reached during the breeding season only some time after August. This point is under investigation.

In Fig. 3, the results obtained by Shapiro and Shapiro in 1934 on seasonal changes in the gonad ratio are plotted on the same graph as the curve relating response per cent. to OD. 50.
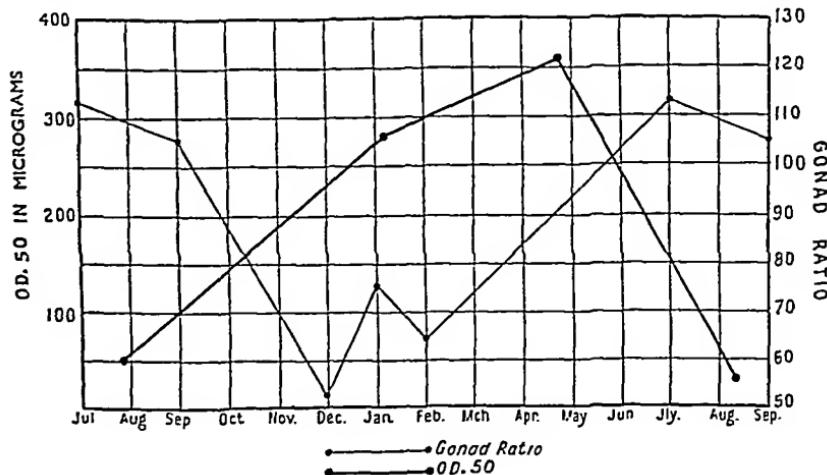
FIG. 3. Relation between OD. 50 and the gonad ratio.

Comparison of the two graphs shows that the sensitivity of the ovaries estimated physiologically in the present investigation correlates reasonably closely with the seasonal changes in the gonad ratio. As the mass of the ovaries decreases relative to the body-weight, the value for OD. 50 increases. The points for maximum gonad ratio and for maximum sensitivity in the two curves are not, however, opposite each other, as that for the gonad ratio is reached in July, whereas OD. 50 is lowest, i.e. ovarian sensitivity is greatest, in August in the present investigation. It is not possible to attach too much importance to this point of difference as the data plotted are for different years. Zwarenstein [1938], however, observed a similar lack of correlation in the case of progesterone.

From the results obtained it is clearly possible to assay solutions of methyl testosterone quantitatively. The construction of similar curves for testosterone, the free hormone, would make it possible to assay these substances more rapidly and simply than can be done by any of the known mammalian techniques. With this object in view the activity of the esterified form of the hormone, testosterone propionate, which is in common use clinically is also being investigated. Zwarenstein [1938] has shown that progesterone can also be assayed in this manner. The

requirements of a reliable method of biological assay include simplicity, rapidity, and the use of large numbers of animals. These conditions are readily fulfilled with *Xenopus* as the test object. With this method of comparison of unknown solutions with a standard preparation, however, it would be necessary to construct monthly curves to take into account the seasonal changes in ovarian sensitivity described.

SUMMARY

1. The capacity of methyl testosterone to induce ovulation in *Xenopus laevis* has been investigated quantitatively in July 1937 and in January, April, and August 1938. At least 40 animals were injected at each dose level.

2. When the response per cent. is plotted against the dose injected, a characteristic curve is obtained in each of the four experiments.

3. The logarithm of the dose in micrograms when plotted against the response per cent. gives a linear relation in all four experiments.

4. Curves obtained for experiments done in between the breeding seasons are displaced to the right, are not as steep as and rise more gradually to a maximum than curves obtained for experiments performed during the breeding season.

5. The sensitivity of the ovaries as indicated by the dose required to produce ovulation in 50% of the animals is elevenfold greater during the breeding season than in between breeding seasons.

6. The minimum effective dose, i.e. the least dose capable of producing any response at all, is elevenfold smaller during the breeding season than it is in between breeding seasons.

7. The seasonal changes in OD. 50 correlate reasonably well with the seasonal changes in the gonad ratio.

8. The curves obtained provide a basis for a simple and rapid method of quantitatively assaying steroids which are gonadotrophic in *Xenopus laevis*.

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THE EFFECTS OF EXTRACTS OF PREGNANT MARE SERUM AND HUMAN PREGNANCY URINE ON THE REPRODUCTIVE SYSTEM OF HYPOPHYSECTOMIZED MALE RATS

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EVANS, Meyer, and Simpson [1933] reported that in male hypophysecto-mized rats an extract of pregnant mare serum was capable of causing regeneration of atrophied testes and resumption of spermatogenesis and fertility. The accessory organs grew to normal size. Histologically the testes of the injected animals appeared normal. Similar results have been achieved with extracts of human pregnancy urine by Smith and Leonard [1934] and Evans, Pencharz, and Simpson [1934]. In the present experiments the relative efficiency of these two types of gonadotrophic extract have been compared in their ability to maintain and restore the reproductive system of hypophysectomized male rats.

METHODS

Male albino rats of the Wistar Institute strain, $2\frac{1}{2}$ to 3 months old, weighing 150 to 190 g. were hypophysectomized by a modified Selye technique [Collip, Selye, and Thomson, 1933]. Criteria of complete hypophysectomy were body-weight curve, adrenal weight, and examination of sella turcica at autopsy. In doubtful cases the sella was sectioned for histological study. Cases of incomplete hypophysectomy are not included in this series.

In one group of rats treatment was commenced immediately after hypophysectomy, and in the second and third series 2 weeks and 4 weeks respectively were allowed to elapse before treatment was started. The treatment was continued for 4 to 6 weeks. In another series no treatment was given, but autopsies were performed at various intervals after operation to ascertain the rate of atrophy of the reproductive organs.

The pregnant mare serum extract was Antex Leo, assayed on immature mice, one unit being described as the amount of the extract which doubles the weight of the ovaries. The pregnancy urine preparation was Physex

Leo, also assayed on immature mice with a unit defined as the amount causing the development of corpora lutea in 50% of the animals. The assay of these extracts has been described by Hamburger and Pedersen-Bjergaard [1937].

The extracts were dissolved in normal saline and made up in solutions containing 10 mouse units per c.c. The animals were injected subcutaneously once daily. Two dosage levels were employed in each series. In immediate treatments these were 0·5 and 1·0 m.u., and in delayed treatments 1·0 and 5·0 m.u. respectively. Each of the treated males was mated with two normal females from 4 weeks after the commencement of the injections to the 6th week.

At autopsy, the testes, prostate, seminal vesicles (with contents), and adrenals were dissected and weighed. The testes and tail and head of epididymis were examined microscopically for spermatozoa and their approximate numbers and motility. The tissues were fixed and studied histologically.

RESULTS

Hypophysectomized controls. The results on untreated hypophysectomized animals are shown in Table I.

Table I. *Rate of atrophy of reproductive system of adult male rats after hypophysectomy*

Time after operation	No. of rats	Body-weight at autopsy	Organ weights per 100 g.				Spermatozoa epididymis		
			Pro-state	Seminal vesicles	Testes	Adrenals	Head	Tail	Testis
Weeks		g.	mg.	mg.	mg.	mg.			
Unoperated	11	158–80	196	149	1269	17·9	+++	+++	+++
1	4	107–40	120	52	1204	12·6	±	+	+++
2	4	112–30	109	56	854	12·6	±	±	++
3–4	5	113–56	87	42	366	8·8	0	0	0
6	2	140	54	34	273	5·7	0	0	0
10	1	109	81	40	258	7·3	0	0	0

The seminal vesicles and prostate degenerated very rapidly after operation. In 1 to 2 weeks they were approximately half the size of unoperated controls. Further degeneration occurred more slowly, and in 6 weeks it was almost complete. The decline in testicular weight was somewhat more gradual, but by 6 weeks it also reached a steady low level. Likewise there was a progressive decline in the adrenal weight until 6 weeks, when the lowest level was reached. Spermatozoa began to disappear from the head of epididymis within the 1st week, and from the tail of epididymis within the 2nd week of operation. By the 3rd to 4th week no spermatozoa could be found either in the epididymis or testis.

Pregnant Mare Serum

Immediate treatment. As shown in the first part of Table II, treatment with Antex 0·5 or 1·0 m.u. daily, maintained the testis weight for the duration of the experiment, namely, 42 days, and spermatogenesis went on unabated. Fertile mating resulted in three rats treated with 1 m.u. daily, although no litters were sired by rats receiving 0·5 m.u. daily. The substitution therapy with Antex at a dosage of 1 m.u. daily was therefore

Table II. *Results of treatment with pregnant mare serum extract (Antex) in adult male hypophysectomized rats*

HRt No.	Daily dose m.u.	Duration Days	Body-weight At operation g.	At autopsy g.	Prostate mg.	Seminal vesicles mg.	Testes mg.	Adrenals mg.	Head epididymis	Tail epididymis	Testis	Result mating
						Organ weights per 100 g.				Spermatozoa		
Immediate treatment												
332	0·5	6	169	133	287	212	1443	19·5	+++	+++	+++	—
335	0·5	7	169	132	168	56	1218	12·1	+++	+++	+++	—
331	0·5	11	149	113	225	230	1490	10·6	+++	+++	+++	—
402	0·5	14	174	120	203	85	1484	10·0	+++	+++	+++	—
336	0·5	42	188	160	80	38	1150	5·0	+++	+++	+++	0
365	0·5	42	190	150	690	948	1030	5·3	+++	+++	+++	0
401	0·5	42	187	157	436	574	1130	5·7	+++	+++	+++	0
333	1·0	11	169	130	265	253	1305	12·3	+++	+++	+++	—
334	1·0	11	154	124	292	348	1730	10·4	+++	+++	+++	—
404	1·0	18	158	119	384	504	1550	10·0	+++	+++	+++	—
337	1·0	42	167	133	262	248	1110	6·0	+++	+++	+++	+
367	1·0	42	174	132	321	355	1040	4·5	+++	+++	+++	+
403	1·0	42	196	168	510	832	1100	4·2	+++	+++	+++	+
Treatment delayed 2 weeks												
357	1·0	28	160	128	73	30	410	5·5	0	0	0	—
358	1·0	28	168	148	115	111	426	5·4	0	0	0	—
391	1·0	28	193	158	243	253	670	6·9	0	0	+	—
390	1·0	28	172	146	336	463	638	4·8	++	++	++	—
17	5·0	28	198	130	900	980	820	4·6	++	+++	+++	—
18	5·0	42	193	140	143	74	315	4·3	0	0	0	0
200	5·0	42	183	128	677	610	860	6·2	+	+	+	0
Treatment delayed 4 weeks												
351	1·0	28	177	151	75	49	290	6·6	0	0	0	—
352	1·0	28	176	122	105	40	219	5·7	0	0	0	—
354	1·0	28	175	138	440	427	560	4·4	0	0	+	—
406	5·0	15	172	154	394	744	556	8·4	0	0	0	—
397	5·0	21	166	139	302	1020	478	5·0	0	0	0	—
394	5·0	26	164	125	705	1250	595	5·6	0	0	0	—
400	5·0	42	185	143	1052	1023	829	4·2	++	++	++	0
78	5·0	42	198	154	493	431	860	3·9	+	++	+	0
53	5·0	42	156	119	103	69	644	5·9	0	0	0	0

Result of mating: + = normal litters sired, 0 = no pregnancy, — = not mated.

complete as far as the spermatogenic function was concerned. The acces
sory organs were stimulated so that their weights at the end of 6 week
treatment were much greater than those of normal untreated controls, the
seminal vesicles being distended with secretion. Histologically the semi
niferous tubules appeared to be normal with slight increase, in some case
of interstitial tissue.

Delayed treatment. When treatment was started 2 weeks after hypo
physectomy when considerable degeneration had taken place, Antex 1 m.u.
daily was not sufficient to bring about much repair although post-opera
tive atrophy was somewhat slowed, especially that of the accessories
Increasing the dosage to 5 m.u. per day brought about considerably
greater repair to the testes and marked stimulation to the prostate and
seminal vesicles in two (HRt. No. 17 and 200) out of the three rats thus
treated. Spermatozoa were present in the testes and epididymis, although
mating was sterile. Histologically spermatogenesis was in evidence, but
the seminiferous tubules were smaller than normal. The interstitial
tissue showed marked proliferation.

The results of therapy started 4 weeks after hypophysectomy were not
essentially different from those where treatment was initiated 2 weeks
after operation. At the dosage level of 1 m.u. per day one rat (HRt. 354)
was restored somewhat in testicular weight and stimulated in the growth
of the accessories, but the other two showed no effect. However, when the
dosage was increased to 5 m.u. daily, greater restoration of the repro
ductive organs occurred. In this group three rats were treated for 15 to 26
days, and three rats for 42 days. The latter receiving the longer period of
therapy showed greater testicular weight with reappearance of spermato
genesis in the majority of instances, although the stimulation of the acces
sories was approximately of the same degree. Pregnancy did not occur in
any of the females mated with the rats of this group. In the sections of
testes from rats showing marked stimulation of the accessory sex glands,
striking interstitial cell proliferation was again in evidence, as shown in
Fig. 1.

Pregnancy Urine Extract

Immediate treatment. Physex at the dosage of 0·5 or 1·0 m.u. per day
was ineffective in maintaining the reproductive system of hypophysecto
mized male rats. At the end of 42 days treatment (Table III) the testes
weighed on the average but little more than those of the untreated oper
ated controls at 3 to 4 weeks (Table I). Spermatogenesis was absent. The
secondary sex organs, though somewhat heavier than those of the un
treated operated controls, remained atrophic.

Delayed treatment. The data on rats receiving therapy 2 weeks after

hypophysectomy were not complete (Table III). Only one animal was available, which received 1 m.u. of Physex daily for 37 days. The results showed no significant degree of restoration to the reproductive system. In the series treated with 5 m.u. daily the response of the testes was more

Table III. *Results of treatment with pregnancy urine extract (Physex) in adult male hypophysectomized rats*

	Treatment		Body-weight		Organ weights per 100 gm.				Spermatozoa			
HRt No.	Daily dose m.u.	Duration Days	At operation g.	At autopsy g.	Prostate mg.	Seminal vesicles mg.	Testes mg.	Adrenals mg.	Head epididymis	Tail epididymis	Testis	Result of mating
					Immediate treatment							
14	0·5	6	168	128	320	265	1280	18·7	+++	+++	+++	—
13	0·5	14	155	106	380	539	1754	11·3	+++	+++	+++	—
84	0·5	42	161	141	117	75	470	5·7	0	0	0	0
193	0·5	42	181	140	101	48	281	5·0	0	0	0	0
194	0·5	42	186	142	122	55	428	7·0	0	0	0	0
16	1·0	5	172	138	416	501	1561	20·2	+++	+++	+++	—
85	1·0	42	153	145	95	59	324	5·5	0	0	0	0
86	1·0	42	165	135	130	83	578	5·9	0	0	0	0
					Treatment delayed 2 weeks							
196	1·0	37	160	88	141	111	313	6·8	0	0	0	0
87	5·0	7	165	147	340	422	1437	8·0	+++	+++	+++	—
190	5·0	17	166	98	265	418	925	8·2	0	0	0	—
88	5·0	42	175	128	181	83	931	8·6	+	++	++	+
192	5·0	42	178	124	113	91	306	4·0	0	0	0	0
					Treatment delayed 4 weeks							
2	1·0	5	183	144	175	102	291	6·9	0	0	0	—
1	1·0	39	184	118	157	75	492	4·2	0	0	0	0
81	1·0	42	186	135	121	68	252	3·7	0	0	0	0
82	1·0	42	173	154	73	30	133	3·9	0	0	0	0
11	5·0	33	150	118	160	68	404	5·9	0	0	0	0
7	5·0	42	154	125	177	126	1126	5·6	+++	+++	+++	+
8	5·0	42	164	132	276	108	1238	3·8	+++	+++	+++	+
79	5·0	42	198	150	174	74	1008	6·7	+	+·	++	0

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marked. In three instances out of four the weight was greater than that of untreated operated controls at 2 weeks, and in two instances spermatogenesis was restored; and in one case, mating was fertile. The accessories exhibited definite stimulation in the rats given a shorter period of treatment, but regression took place in those given 42 days of therapy. This suggests that Physex in adequate doses stimulates the accessory sex glands initially, but in continued administration this effect is lost, allowing regression to take place.

When therapy was delayed 4 weeks after hypophysectomy 1 m.u. a day

complete as far as the spermatogenic function was concerned. The accessory organs were stimulated so that their weights at the end of 6 weeks treatment were much greater than those of normal untreated controls, the seminal vesicles being distended with secretion. Histologically the seminiferous tubules appeared to be normal with slight increase, in some cases of interstitial tissue.

Delayed treatment. When treatment was started 2 weeks after hypophysectomy when considerable degeneration had taken place, Antex 1 m.u. daily was not sufficient to bring about much repair although post-operative atrophy was somewhat slowed, especially that of the accessories. Increasing the dosage to 5 m.u. per day brought about considerably greater repair to the testes and marked stimulation to the prostate and seminal vesicles in two (HRt. No. 17 and 200) out of the three rats thus treated. Spermatozoa were present in the testes and epididymis, although mating was sterile. Histologically spermatogenesis was in evidence, but the seminiferous tubules were smaller than normal. The interstitial tissue showed marked proliferation.

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194	0·5	42	186	142	122	55	428	7·0	0	0	0	0
16	1·0	5	172	138	416	501	1561	20·2	+++	+++	+++	—
85	1·0	42	153	145	95	59	324	5·5	0	0	0	0
86	1·0	42	165	135	130	83	578	5·9	0	0	0	0
					Treatment delayed 2 weeks							
196	1·0	37	160	88	141	111	313	6·8	0	0	0	0
87	5·0	7	165	147	340	422	1437	8·0	+++	+++	+++	—
190	5·0	17	166	98	265	418	925	8·2	0	0	0	—
88	5·0	42	175	128	181	83	931	8·6	+	++	++	+
192	5·0	42	178	124	113	91	306	4·0	0	0	0	0
					Treatment delayed 4 weeks							
2	1·0	5	183	144	175	102	291	6·9	0	0	0	—
1	1·0	39	184	118	157	75	492	4·2	0	0	0	0
81	1·0	42	186	135	121	68	252	3·7	0	0	0·	0
82	1·0	42	173	154	73	30	133	3·9	0	0	0	0
11	5·0	33	150	118	160	68	404	5·9	0	0	0	0
7	5·0	42	154	125	177	126	1126	5·6	+++	+++	+++	+
8	5·0	42	164	132	276	108	1238	3·8	+++	+++	+++	+
79	5·0	42	198	150	174	74	1008	6·7	+	+·	++	0

Result of mating: + = normal litters sired, 0 = no pregnancy, — = not mated.

marked. In three instances out of four the weight was greater than that of untreated operated controls at 2 weeks, and in two instances spermatogenesis was restored; and in one case, mating was fertile. The accessories exhibited definite stimulation in the rats given a shorter period of treatment, but regression took place in those given 42 days of therapy. This suggests that Physex in adequate doses stimulates the accessory sex glands initially, but in continued administration this effect is lost, allowing regression to take place.

When therapy was delayed 4 weeks after hypophysectomy 1 m.u. a day

was likewise ineffective in bringing about any significant repair in the atrophied testes and secondary sex glands, but a daily dose of 5 m.u. was much more efficient. In the latter group of four rats, three showed at the end of 6 weeks' treatment testicular weights approaching those of normal controls, and active spermatogenesis (Fig. 2). Fertile mating occurred in two of the rats. Of the secondary sex glands, the prostate was approximately the same in weight as the unoperated controls, while the seminal vesicles were somewhat smaller. The substitution therapy therefore was apparently complete from the viewpoint of spermatogenesis, but not so when the accessory glands were considered. An initial stimulation of the accessories followed by regression may have occurred, as shown in the group of rats treated 2 weeks after operation with 5 m.u. a day for a varying number of days.

DISCUSSION

From the above results on the rat it is clear that both the extract of pregnant mare serum and pregnancy urine have a stimulating action, but they differ in several important respects. In maintenance experiments when treatment commenced immediately after hypophysectomy, Antex in daily doses of 0·5 or 1·0 m.u. was able to sustain spermatogenesis and stimulate interstitial cell function for as long as 42 days after ablation of the pituitary while similar doses of Physex were ineffective. Thus in contrast to Antex, 1 m.u. of Physex (assayed by a different technique) does not contain sufficient gonadotrophic substance to produce significant effects on the reproductive system of hypophysectomized male rats. Higher dosage of Physex would probably be effective, as shown in reparative experiments.

In reparative treatment, Antex in 5 m.u. daily dose was more effective as an interstitial cell stimulator, but less efficient in restoring spermatogenesis than Physex in similar dosage. Thus Antex-treated rats showed greater prostate and seminal vesicle weights, but smaller testes than Physex-treated animals. While mating of Antex-treated rats did not result in any litters, it was fruitful in some of the Physex-treated animals. Increasing the dosage of Physex from 1 to 5 m.u. brought its effect on spermatogenesis from a position of relative ineffectiveness to one of marked potency; while a similar increase in the dosage of Antex failed to produce any greater effect in restoring than in maintaining spermatogenic function. This suggests that the difference between the dose required in maintenance and that in restoration is greater in the case of Antex than in Physex.

The relative lack of interstitial cell stimulating action in Physex may only be apparent rather than real, as it is possible that shorter periods of

treatment produced larger prostate and seminal vesicle weights, whereas after 6 weeks treatment, regression in size had taken place. However, in the spermatogenic activity of either Physex or Antex there is no evidence of initial stimulation followed by subsequent regression in the response of the testis.

In the experiments of Evans *et al.* [1933] on the action of pregnant mare serum on hypophysectomized male rats, the interval between operation and injection varied between 29 and 41 days and the treatment was continued for 14 to 84 days. This corresponds to the delayed treatment described. The stimulation of the interstitial cells and restoration of spermatogenic function has been confirmed, but the latter was apparently not complete in that no fertile mating occurred. This failure may be related to the dosage employed. At a higher dosage level it is possible that enough enhancement of spermatogenesis may occur as to result in fertile mating. It must be pointed out, however, that the secondary effect of the androgens which are liberated in response to Antex may be an important factor in maintaining spermatogenesis. Cutuly, McCullagh, and Cutuly [1937 and 1938] showed that chemically pure androsterone and testosterone would maintain the tubules of the testes after hypophysectomy, if injection were started immediately post-operative. Once the testes were atrophied, however, the androgens would not cause repair of the tubules. The observed findings that Antex would maintain spermatogenesis if injections were started immediately after hypophysectomy, but did not restore spermatogenesis to a point where fertile matings occurred when therapy was delayed for 2 to 4 weeks, could be explained on the experimental findings of Cutuly *et al.* However, since there was definite histological evidence of spermatogenesis in some cases and a definite increase in the weight of the testes, it would seem that Antex produced a direct action on the testes in the delayed treatment experiments. The secondary liberation of androgens in the immediate experiments is probably an accessory factor in maintaining spermatogenesis.

Smith and Leonard [1934] noted that in hypophysectomized male rats, treatment with pregnancy urine extract produced an enlargement of the accessory reproductive organs and hypertrophy of the interstitial cells, but by the 30th day of treatment there was a marked regression of the accessories and the interstitial tissue. Likewise Greep and Fevold [1937] showed that in adult hypophysectomized rats partial regression of the accessory organs occurred after 30 to 40 days of treatment with luteinizing hormone of the pituitary. In the results described a similar phenomenon was apparent so that by the 42nd day of treatment with Physex the prostate and seminal vesicles were either atrophic or only slightly restored. In the experiments of Smith and Leonard they were

unable to restore completely the spermatogenic function of their animals when injections of pregnancy urine extract were begun after a lapse of 20 to 75 days following operation. In the experiments described above the restoration with a similar preparation seemed complete in that fertility returned in some of the animals treated with a daily dose of 5 m.u. starting from 2 or 4 weeks after hypophysectomy.

SUMMARY

The gonadotrophic effects of extracts of pregnant mare serum and pregnancy urine were compared in adult male hypophysectomized rats. When treatment was instituted immediately after operation small doses of pregnant mare serum extract were adequate in stimulating interstitial cells with resulting enlarged accessory reproductive organs, and in maintaining spermatogenesis so that mating was fertile. Pregnancy urine extract in similar doses was ineffective. When treatment was delayed for 14 or 28 days after operation, but given in 5 or 10 times the dosage used in immediate treatment, pregnant mare serum seemed to be a more efficient interstitial cell stimulator than pregnancy urine, but the reverse was true in regard to the spermatogenic effect.

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Fig. 1. Testis of rat (HRt 400) treated with 5 m.u. Antex daily for 42 days starting
4 weeks after hypophysectomy. × 190

Fig. 2. Testis of rat (HRt 8) treated with 5 m.u. Physex daily for 42 days
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THE EFFECTS OF EXTRACTS OF PREGNANT MARE SERUM AND HUMAN PREGNANCY URINE ON THE REPRODUCTIVE SYSTEM OF HYPOPHYSECTOMIZED FEMALE RATS

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IN a preceding paper [Liu and Noble, 1939] it has been shown that in male hypophysectomized rats, extracts of both pregnant mare serum and pregnancy urine were capable of exerting a marked effect on the testes as well as on the accessory sex organs. In the present experiments it was attempted to determine how far the results obtained on the male were comparable with those on the female hypophysectomized rats under similar experimental conditions.

METHODS

The experimental procedure was the same as previously reported [1939]. Vaginal smears were made daily to determine the oestrogenic activity. In mating experiments, normal males were placed with the hypophysectomized females at 2 weeks after the commencement of treatment.

RESULTS

Hypophysectomized controls. As shown in Table I, the rate of atrophy of ovaries and uteri of hypophysectomized rats was gradual up to 6 weeks after operation. After that time very little further decline in weight occurred. The vaginal smear showed a dioestrus condition within 3 to

Table I. *Rate of atrophy of sex organs of female rats after hypophysectomy*

Time after hypophysectomy Weeks	No. of rats	Body-weight at autopsy g.	Organ weights per 100 g. body-weight		
			Ovaries mg.	Uteri mg.	Adrenals mg.
Unoperated	6	145–73	28·7	240	32·5
1	4	126–44	24·3	142	28·8
2	6	120–68	22·5	129	23·2
3–4	4	114–36	17·0	117	14·2
6	3	136–67	15·0	82	8·9
8	2	146–53	15·8	78	6·4
10	2	137–60	9·0	63	6·7

[1] On leave from Peiping Union Medical College, Peiping, China.
[2] Work performed during the tenure of a Leverhulme Fellowship of the Royal College of Physicians, London.

4 days after hypophysectomy; histologically the follicles rapidly degenerated and disappeared, but corpora lutea remained for relatively long periods. Even after 10 weeks, corpora lutea were still discernible, although much fibrous tissue replacement had taken place.

Pregnant Mare Serum Extract

Immediate treatment. When treatment was given immediately after hypophysectomy 0·5 m.u. of Antex daily maintained the ovarian weight and increased the uterine weight for 24 to 34 days (Table II). Vaginal oestrus continued for the duration of the experiment. However, in one animal (HRt 259), in which treatment was carried on for 42 days, the ovaries and uterus were markedly atrophied and cessation of vaginal oestrus occurred by the 27th day of treatment. Five rats treated for 12

Table II. *Results of treatment with pregnant mare serum extract (Antex) in female hypophysectomized rats*

	Treatment		Body-weight		Organ weights per 100 g.			Oestrus after treatment		
HRt no.	Daily dose m.u.	Duration Days	At operation g.	At autopsy g.	Ovaries mg.	Uterus mg.	Adrenals mg.	Onset Days	Cessation Days	Duration Days
				Immediate treatment						
172	0·5	24	200	147	30·6	246	14·3	7	no	17+
170	0·5	33	192	138	34·8	538	10·9	6	no	27+
173	0·5	34	177	120	32·5	550	11·7	6	no	28+
259	0·5	42	172	147	14·3	108	7·5	6	27	21
174	1·0	12	173	120	33·3	391	28·3	5	no	7+
260	1·0	19	160	104	31·7	435	17·3	5	no	14+
211	1·0	21	178	136	28·7	257	16·9	5	no	16+
175	1·0	23	195	113	33·6	410	15·0	5	no	18+
212	1·0	23	183	149	20·1	326	17·4	5	no	18+
				Treatment delayed 2 weeks						
161	1·0	23	180	125	22·4	267	10·4	6	no	17+
162	1·0	32	160	126	19·8	274	13·5	6	no	26+
157	1·0	47	180	138	23·2	308	11·6	6	no	41+
213	5·0	10	169	133	24·8	287	13·5	4	no	6+
135	5·0	11	182	128	47·7	365	19·5	4	no	7+
133	5·0	15	161	129	39·5	308	11·6	4	no	11+
214	5·0	34	191	136	19·9	305	8·1	4	no	30+
132	5·0	35	187	122	32·8	500	11·5	5	no	30+
				Treatment delayed 4 weeks						
109	1·0	42	177	145	9·2	157	6·9	6	no	36+
112	1·0	42	150	111	24·3	273	7·2	5	no	37+
113	1·0	42	155	109	24·8	297	7·3	5	no	37+
108	5·0	16	175	118	39·8	415	9·3	5	no	11+
107	5·0	38	184	136	21·3	280	9·6	5	no	33+

to 23 days had an approximately normal ovarian weight and distinctly heavier uterine weight than the unoperated controls (Table I). Vaginal oestrus was maintained for the entire period. The histological sections showed many large corpora lutea, with a few well developed follicles.

Delayed treatment. When Antex therapy was started 2 weeks after hypophysectomy and given in 1 m.u. daily dosage for 23 to 47 days, some regression in ovarian weight was found, but uteri were larger than normal. At a dosage of 5 m.u. per day, treatment for 10 to 15 days in three rats resulted in larger ovaries than normal, while treatment for 34 to 35 days in two other rats gave ovarian weights approaching normal, but less than those treated for the shorter period. Uteri were larger than normal in all cases, and vaginal oestrus always continued for the duration of the experiment.

When therapy was not instituted until 4 weeks after operation 1 m.u. per day of Antex for 42 days was apparently not sufficient to produce normal ovarian weight, although normal uterine weight was maintained. When the dosage was increased to 5 m.u. daily, one rat treated for 16 days gave larger ovaries and the other treated for 38 days gave somewhat smaller ovaries than normal. Uteri in both cases were large and vaginal oestrus was continuous for as long as 37 days.

Mating occurred in three animals in this series as shown by the presence of vaginal plugs, but no pregnancy followed.

Histologically the larger ovaries associated with the shorter period of treatment showed many well-formed corpora lutea with occasional fully developed follicles, but the smaller ovaries, from rats treated for the longer period, exhibited regressing corpora lutea and a few primordial follicles. Interstitial stroma appeared to be increased.

Pregnancy Urine Extract

Immediate treatment. As shown in Table III, Physex therapy started immediately after hypophysectomy in 0·5 m.u. daily doses maintained the ovarian and increased the uterine weight in the rats treated up to 22 days. One rat in the series receiving the treatment for 43 days showed marked regression in weight of both ovaries and uterus. The group receiving 1 m.u. daily consisted of nine rats treated for a period varying from 5 to 42 days. The ovarian and uterine weights showed an inverse relationship with the duration of treatment, indicating that there was an initial stimulation followed by subsequent regression. Likewise the animals exhibited continuous vaginal oestrus shortly after the commencement of treatment, but within 14 to 19 days oestrus gave place to dioestrus in spite of continued treatment. The state of anoestrus was usually associated with smaller ovaries and uteri. Histologically corpora lutea were well developed with a few atretic follicles.

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HRt no.	m.u.	Days	g.	g.	mg.	mg.	mg.	Days	Days	Days
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remained stimulated and oestrus was continuous throughout the experimental period. Mating took place, but no pregnancy followed.

Pregnancy urine extract maintained or restored ovarian weight, but regression on prolonged treatment was more marked and took place rapidly. A regression in uterine weight followed after initial stimulation. Vaginal oestrus was maintained only for a short period despite continued treatment. Mating did not occur.

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predominating luteinizing action, especially in cases where treatment was prolonged.

Both extracts consistently induced continuous vaginal oestrus in hypophysectomized rats, although the duration of such differed between the two preparations. In Antex-treated animals oestrus, once initiated, was maintained in all but one for the whole of the experimental period up to 6 weeks. In Physex-treated animals, however, oestrus lasted for a period of only 9 to 21 days and did not reappear despite continued treatment. The continuous vaginal oestrus was associated with an increased uterine weight. Thus with Antex treatment, which maintained continuous vaginal oestrus up to the expiration of the experiments, uterine weights were either normal or greater than normal. With Physex treatment, which allowed the animals to pass into dioestrus within the experimental period, the uteri became atrophied. Antex, therefore, was effective in stimulating the production of sufficient oestrogen to give rise to enlarged uteri and continuous oestrus. On the other hand, the effects of Physex on the uterus and vagina were much shorter in duration. Greep [1938] under similar experimental conditions has shown that a correlation existed between the number of corpora lutea in the ovary and the duration of oestrus. The results described seem to support such an idea in that the animals which did not continue in oestrus had atrophic ovaries at autopsy, though even after the ovaries had become atrophic a month after hypophysectomy the animal still showed an oestrus response. This transitory effect of pregnancy urine extract in the female would appear to be similar to that which was found to occur in the male, and the possibility of antihormone formation must be considered. Mating took place occasionally in the Antex-treated animals, but in no case in the Physex-treated animals. Pregnancy did not occur in any of the treated animals; a similar failure has previously been noted by Evans, Meyer, and Simpson [1933] in the treatment of hypophysectomized female rats with a pregnant mare serum extract. The female reproductive system would appear to be so regulated that Antex or Physex, though adequate for the male under certain conditions do not constitute complete replacement therapy in the female, not at least under the experimental conditions reported.

SUMMARY

A series of female hypophysectomized rats were treated with extracts of pregnant mare serum or pregnancy urine. Treatment either immediately after hypophysectomy with small doses or delayed 2 or 4 weeks after operation with larger doses gave similar findings. With the pregnant mare serum extract the ovarian weight was maintained or restored for a time, but prolonged treatment resulted in slight regression. Uteri

remained stimulated and oestrus was continuous throughout the experimental period. Mating took place, but no pregnancy followed.

Pregnancy urine extract maintained or restored ovarian weight, but regression on prolonged treatment was more marked and took place rapidly. A regression in uterine weight followed after initial stimulation. Vaginal oestrus was maintained only for a short period despite continued treatment. Mating did not occur.

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COMPARATIVE EFFECTS OF CERTAIN GONADOTROPHIC EXTRACTS ON THE OVARIES OF NORMAL AND HYPOPHYSECTOMIZED RATS

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ONE of the methods most commonly used in the biological assay of gonadotrophic extracts is the quantitative measurement of the response elicited in the ovaries of the immature rat. This method, used on account of its convenience, suffers from the disadvantage that the animal's own pituitary gland is present and must be regarded as a potential source of complication in the assay results. Experience has shown that the complication may be quantitative or qualitative or both. Smith [1930] found that the difference in response of normal and hypophysectomized rats to injections of gonadotrophic extracts of the pituitary gland was quantitative rather than qualitative, whereas the difference in the response of the ovaries of normal and hypophysectomized rats to extracts of urine of pregnant women (luteinizing hormone) and of the urine of ovariectomized women (follicle stimulating hormone) has been found to be most definitely qualitative [Selye, Collip, and Thomson, 1933; Leonard and Smith, 1934]. Tyndale, Levin, and Smith [1938] have recently demonstrated that the amount of gonadotrophic extract from the urine of post-menopausal women required to cause luteinization of the ovary of the hypophysectomized rat is 8 to 10 times the amount necessary in the normal immature rat.

It is evident, therefore, that in such tests carried out on the normal immature rat, the stimulation of the gonads is contributed to by endogenous gonadotrophic hormones. It is unlikely that the amount of hormone normally present in the immature rat is sufficient, even by synergism, to contribute to ovarian stimulation, so that the extract which is injected presumably stimulates the pituitary gland to secrete additional amounts of hormone. This stimulation may perhaps be the result of a direct action of the injected hormone on the pituitary gland, but it seems more likely

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that the violent changes caused in the ovary of the immature animal may lead to secondary stimulation of the pituitary gland, which attempts to respond in the same way as it may be supposed to do during the normal cycle. Thus from the work of Selye, Collip, and Thomson [1935], Hohlweg [1936], and Fevold, Hisaw, and Greep [1936] it is established that luteinization of the ovary of a normal rat, under the influence of a follicle stimulating extract, is caused by the secretion of luteinizing hormone from the pituitary gland, in response to excessive output of oestrogen by the stimulated ovary.

The recognition of the complications introduced by such effects is obviously of major importance in the determination, by biological assay, of the qualitative difference between various types of gonadotrophic activity. The work reviewed above has, therefore, been extended by a comparative study of the action of gonadotrophic extracts from the pituitary gland of various species, including man, as tested not only on the intact immature rat but also on the hypophysectomized rat.

The complications due to the presence of the pituitary gland in the test animals have been further studied in relation to the apparently selective reactions between various gonadotrophic extracts and antigonadotrophic sera. Collip, Selye, and Williamson [1938] found that prolonged administration of an extract of pig pituitary gland to the intact rat produced a condition in which the animal responded to extract of pregnancy urine in the same way as the hypophysectomized rat. This result implies that the antigonadotrophic activity evoked by prolonged treatment with pig pituitary gland (*a*) is able to neutralize the endogenous gonadotrophic hormones of the rat, which do not therefore affect the response of the ovary to the urine extract, and (*b*) is unable to neutralize the extract from human urine of pregnancy. The positive result obtained by Rowlands [1938 *a*], who observed that an extract of human urine of pregnancy was neutralized by antiserum to an extract of ox pituitary gland, might therefore have been due to the use of intact test animals, since the capacity of the antiserum to neutralize rat gonadotrophic hormones would, in any case, obliterate the greater part of the increase in ovarian weight, normally caused in immature rats by injecting the gonadotrophic substance from the urine of pregnant women.

Similarly, the amount of anti-luteinizing serum required to convert the response of the rat ovary to an extract of gelding pituitary gland into pure follicular growth [Rowlands, 1938 *b*] may well have been materially affected by the secretion of luteinizing substance by the test animal's own pituitary gland, in response to the extensive follicular growth caused by the extract. Confirmatory experiments, using hypophysectomized rats, have, therefore, been carried out on both these problems.

<center>METHODS</center>

Extracts. The extracts of the pituitary gland which were used included (*a*) horse pituitary—AP70B and AP70D (gelding only) and AP61D; (*b*) pig pituitary—AP63D and AP74D; (*c*) sheep pituitary—AP66D; and (*d*) ox pituitary—AP15B [see Chance, Rowlands, and Young, 1939]. In addition, several human pituitary glands were assayed individually in normal and hypophysectomized rats. These glands were desiccated in acetone, freed from their fibrous capsules, ground in a mortar, and suspended in slightly alkaline water for 2 hours. Most of the glandular tissue dissolves under these conditions and remains in solution on neutralization to pH 7·5. This solution was directly used for injection.

Other sources from which gonadotrophic extracts were prepared included (*a*) urine of pregnant women, UP10; (*b*) serum of pregnant women, PWS77 [see Boycott and Rowlands, 1938]; (*c*) urine of an ovariectomized woman. This extract (CU1A) was prepared by precipitation with alcohol; the precipitate (1 mg. ≡ 0·13 c.c. of urine) was dissolved in water at pH 4·7 from which solution a precipitate was obtained with tannic acid by the method of Thomsen and Pedersen-Bjergaard [1936]. It was purified further by reprecipitating with alcohol from borate buffer at pH 9·3. 1·0 mg. of the final precipitate ≡ 6·4 c.c. of original urine; (*d*) urine of a senile woman. This extract (SU2) was prepared by precipitation with alcohol.

Antisera. Two antisera, obtained from rabbits immunized to extracts of ox pituitary gland, were used—Serum A described by Rowlands [1938 *b*] and Serum B [Rowlands, 1939]. These antisera exert, in the normal immature rat, a differential anti-luteinizing action on the extract of gelding pituitary gland, AP70B.

Hypophysectomy. Immature female rats, weighing 40–50 g., were hypophysectomized, using the retropharyngeal approach. A period of 7–11 days elapsed before treatment, to allow for the disappearance of endogenous gonadotrophic hormone from the circulation and for the consequent atrophy of the ovaries.

Assay. Groups of normal or hypophysectomized immature rats were injected subcutaneously once daily for 5 days. The hypophysectomized rats were weighed at operation, on the day of the first injection, and again after killing. They were killed 24 hours after the last injection; the ovaries and uteri were dissected and fixed in Bouin's fluid. The sella turcica of each rat was examined for any fragments of pituitary tissue, and if any fragment of anterior lobe tissue remained, the animal was discarded from the test. The ovaries and uteri were weighed from 70% alcohol.

Histology. Serial sections of the ovaries were cut at 10 μ; every fifth section was mounted and stained in Mayer's haemalum and eosin. Histo-

logical examination was made of the ovaries of a number of hypophys-ectomized rats in a control series. It was observed that after a period of 7–11 days following the operation the ovaries had undergone definite atrophic changes. Their average weight decreased from 10 mg. at the time of operation to 5–6 mg. 7 to 11 days later. Only very few follicles possessing an antrum were found (Fig. 1), although Lane and Greep [1935] stated that the percentage number of antrum-containing follicles decreased by only 10% during the first 10 days after the operation. The ovum contained in each of the follicles appeared normal.

Comparison of the effects in normal and hypophysectomized rats of gonado-trophic extracts of the pituitary gland

Extracts of the pituitary gland of domestic animals. The data obtained are given in Table I. It is seen that, with the exception of the extracts of gelding pituitary (AP70B and AP70D), the actual increase in the weight of the ovary of the hypophysectomized rat is less than that in the normal rat. Hellbaum [1933] has shown that such extracts contain a high pro-portion of follicle stimulating hormone. With one extract (AP63D) the increase is reduced to one-third. Even so, however, it must be remembered that the increase in weight, in proportion to the original size of the ovary in the hypophysectomized rat, is usually as great as, or may even be greater than, in the normal rat.

Table I. *Assay of various gonadotrophic extracts on normal and on hypo-physectomized rats. The weight of the ovaries of the normal rat = 10 mg.; those of the hypophysectomized rat = 6 mg.*

Species of pituitary	No. of extract	Amount of extract injected	Test animal		Average weight of		Average increase in ovarian weight	Increase in weight
			Condition	No.	Ovaries	Uteri		Weight of control ovaries
	(AP.)	mg.			mg.	mg.	mg.	
Horse	70B	2·5	N	5	54	65	44	4·4
„	„	2·5	H	8	52	64	46	7·7
Horse	70D	2·5	N	5	31	57	21	2·1
„	„	5·0	H	4	61	49	55	4·6
Horse	61D	1·0	N	5	67	84	57	5·7
„	„	1·0	H	3	28	58	22	3·7
Sheep	66D	10·0	N	5	26	45	16	1·6
„	„	12·5	H	8	19	55	13	1·7
Pig	74D	12·5	N	10	25	26	15	1·5
„	„	10·0	H	5	16	51	11	2·3
„	„	25·0	N	10	31	34	21	2·1
„	„	25·0	H	3	23	69	17	2·8
Pig	63D	25·0	N	5	56	58	46	4·6
„	„	25·0	H	4	21	53	15	2·5
Ox	15B	50·0	N	5	15	19	5	0·5
„	„	50·0	H	5	9	34	3	0·5

N = Intact immature rat.
H = Hypophysectomized immature rat.

The ovaries of both normal and hypophysectomized rats which received extract of gelding pituitary gland (Fig. 5) contained (*a*) corpora lutea which, as far as could be determined from histological examination of the ovary, resulted from normal ovulation; (*b*) large follicles showing greater or less degree of luteinization of the membrana granulosa, many of which were becoming solid; and (*c*) medium-sized follicles in which the granulosa had fragmented, but was not luteinized. The ovaries of the rats injected with extracts of pig and sheep pituitary gland (Fig. 6) were also heavily luteinized, but the structures observed were smaller than those seen in the ovaries of the rats that received extracts of gelding pituitary gland. Medium-sized follicles were seen in different stages of luteinization; in some the membrana granulosa was completely luteinized and formed a solid mass of lutein tissue enclosing the ovum. Each of these structures, which had the appearance of a corpus luteum, usually measured only half the diameter of a true corpus luteum. There was thus some sign of follicle stimulation prior to luteinization, but the stimulation of this type was obviously much weaker than that obtained with the extracts of the gelding pituitary gland. Extracts of ox pituitary gland (AP15B) produced little, if any, follicle stimulation in the ovaries of hypophysectomized rats, since only very small follicles were present, which were all luteinized.

Extracts of human pituitary gland. The result of the assays are shown in Table II. The increase in the gonadotrophic activity of the human pituitary gland after the period of sexual activity [Henderson and Rowlands, 1938] is clearly shown. In the previous work on the human pituitary gland it could not be determined, from the tests on normal immature rats, whether the increased content of gonadotrophic hormone in the gland, after the menopause, was associated with any variation in the proportion of follicle-stimulating hormone and luteinizing hormone.

Very striking histological differences are observed in the present experiments, between the response of the ovaries of the normal and of the hypophysectomized rat to some of the human glands. The pituitary extract (HP553) of a senile woman, aged 79 years, caused, in the ovaries of the intact rat, development of follicles which ovulated, giving rise to corpora lutea. The same amount of this gland when injected into hypophysectomized rats caused the growth of a large number of follicles, which failed to ovulate. No corpora lutea were found and no luteinization of the membrana granulosa had occurred. A similar difference in the response between normal and hypophysectomized rats was observed with injections of the pituitary extract (HP737) of a woman aged 67 years (Figs. 7 and 8). The pituitary gland (HP678) from another woman, aged 75 years, when injected into intact rats in a total dose of 1 mg., produced

ovaries having an average weight of 24 mg. Corpora lutea were found in all of these ovaries. The injection of double this amount of extract into each of a group of five hypophysectomized rats produced ovaries having an

Table II. *Assay of extracts of human pituitary glands on normal and on hypophysectomized rats*

No. HP.	Sex	Age Years	Test rats		Amount of dried gland injected mg.	Average weight of ovaries mg.	Number of rats in which luteinization occurred in the ovaries
			Condition	No. per group			
553	♀	79	N	5	1	76	4
,,	,,	,,	H	4	1	28	0
678	♀	75	N	5	1	24	5
,,	,,	,,	H	5	2	40	1
718	♀	68	N	5	1	43	5
,,	,,	,,	H	3	2	73	3
737	♀	67	N	5	1	59	5
,,	,,	,,	H	5	1	31	0
755	♀	65	N	5	1	58	5
,,	,,	,,	H	4	1	57	4
739	♀	62	N	5	1	57	5
,,	,,	,,	H	4	1	44	4
727	♀	61	N	5	1	61	5
,,	,,	,,	H	5	1	56	5
731	♀	37	N	5	5	31	5
,,	,,	,,	H	2	5	24	0
593	♀	35	N	5	5	39	5
,,	,,	,,	H	5	5	34	5
486	♀	34	N	5	5	38	5
,,	,,	,,	H	4	5	47	4
743	♂	81	N	5	2	43	5
,,	,,	,,	H	4	4	75	4
682	♂	76	N	5	3	44	5
,,	,,	,,	H	5	2	10	0
728	♂	61	N	5	2	23	5
,,	,,	,,	H	4	4	35	4
116	♂	55	N	5	2	49	4
,,	,,	,,	H	4	2	24	1

N = Intact immature rats.
H = Hypophysectomized immature rats.

average weight of 40 mg. The response in the ovaries of four of these rats consisted of follicle stimulation without any luteinization; in the remaining animal of the group some slight luteinization of the membrana granulosa had occurred. All these extracts produced a pronounced stimulation of the interstitial or stromal elements of the ovary. Extracts of three other glands, HP755, 739, and 727, from women whose ages were 65, 62, and 61 years respectively, caused in the ovaries of both normal and hypophysectomized rats the same quantitative and qualitative response.

Assays were carried out on three glands, HP731, 593, and 486, from women whose ages were 37, 35, and 34 years respectively. The amount of these glands necessary to produce ovarian stimulation in immature rats is seen to be at least five times as great as that required by the pituitary glands of post-menopausal women. Extracts of the glands (HP593 and 486) caused the formation of corpora lutea in the ovaries of hypophysectomized rats, whereas the third, HP731, when tested on two hypophysectomized rats, produced only follicular stimulation.

Four pituitaries, from men of various ages, were tested, but only one, HP116, from a man aged 55, gave any indication of a qualitative difference between the response given by the control and operated groups.

It is observed that, when the response given to all the human pituitary glands in the hypophysectomized rats consists of follicle stimulation and luteinization, the quantitative response approximates that obtained in the intact rat, given a similar amount of extract. On the other hand, when the response in the hypophysectomized rat consists only of follicle stimulation, the average weight of the ovaries is considerably less than that produced by a similar dose in the normal rat. Nevertheless, the weight of such ovaries may be considerably greater than the minimum weight at which 100% luteinization occurs in intact rats when extract with follicle-stimulating and luteinizing activity is used.

The results suggest that the pituitary gland of the post-menopausal woman contains a greater amount of follicle stimulating hormone than that of a woman during the period of sexual activity, and since it is shown that some luteinizing hormone is present, its secretion is probably not in complete abeyance during the years following the menopause.

Comparison of Effect in Normal and Hypophysectomized Rats of Gonadotrophic Extracts from Extra-pituitary Sources

The results of the assays carried out on these extracts are shown in Table III.

Extracts of urine and serum from pregnant women. An extract of urine of pregnancy (UP10) in an amount (1 mg.) which produces an almost maximum increase in the weight of the ovaries of the normal rat [Deanesly, 1935] produced only a slight increase in the weight of the ovaries of the hypophysectomized rat. The injection of five times this amount of extract caused only little additional increase in weight. The stimulation produced in the ovaries of these rats consisted of luteinization of all small follicles and stromal tissue, such as has been described by Selye, Collip, and Thomson [1933] and Leonard and Smith [1934]. A similar effect (Fig. 2) was obtained in the ovaries of hypophysectomized rats with an extract of serum of pregnant women (PWS77). The similarity found by Boycott

and Rowlands [1938] in the biological properties of gonadotrophic preparations from urine and serum of pregnant women, when tested on normal immature rats, is therefore confirmed.

Extracts of urine from senile and ovariectomized women. Zondek [1930] found that the urine of ovariectomized or post-menopausal women

Table III. *Assay of gonadotrophic extracts obtained from extra-pituitary sources, on normal and hypophysectomized rats*

Extract		Total amount injected mg.	Test Rats		Time of first injection after hypophysectomy	Average weights	
Source	No.		Condition	No.	Days	Ovaries mg.	Uteri mg.
Urine of	UP10	1·0	N	10	—	34	95
pregnant	,,	1·0	H	4	9	12	14
women	,,	5·0	H	4	10	15	36
Serum of	PWS77	2·0	N	10	—	47	102
pregnant women	,,	2·0	H	2	2	13	14
Urine of	CU1A	10·0	N	4	—	23	68
ovariec-tomized woman	,,	10·0	H	4	10	6	14
Urine of	SU2	100·0	N	5	—	15	37
senile woman	,,	100·0	H	4	9	5	13
Serum of	PMS16	1·5	N	10	—	164	100
pregnant mares	,,	1·5	H	5	1	104	97
,,	PMS18	1·0	N	15	—	97	116
,,	,,	1·0	H	5	10	49	116

N = Intact immature rats.
H = Hypophysectomized immature rat.

yielded an extract having follicle-stimulating properties. This observation was confirmed by Leonard and Smith [1934], who demonstrated the effect of such extracts on the ovaries of hypophysectomized rats.

The injection of 100 mg. ($\equiv$ 19 c.c. urine) of an extract of urine (SU2) from a woman aged 79 years produced only a slight increase in the weight of the ovaries of the normal immature rat, although a similar amount had no effect on the ovaries of the hypophysectomized rat.

Ten milligrams ($\equiv$ 64 c.c.) of an extract (CU1A) of urine of an ovariectomized woman produced a good response in the ovaries of normal immature rats. The average ovarian weight was 23 mg.; the response consisted of follicle stimulation followed by a limited amount of corpus luteum formation. No response was shown in the ovaries of the hypophysectomized rats to a similar amount of this extract.

The significance of the fact that the response was obtained only in

intact immature rats and of the discrepancy with the results of Tyndale, Levin, and Smith [1938] is not at present obvious.

Extracts of pregnant mare serum. Two extracts of pregnant mare serum (PMS16 and 18) resembled those of horse pituitary gland in their action on the ovaries of normal and hypophysectomized rats. The injection of PMS16, when begun 24 hours after the removal of the pituitary gland, produced an increase in ovarian weight of about two-thirds that obtained in the intact rat. The increase in the weight of the ovaries produced by the extract PMS18, when injections were begun 10 days after hypophysectomy, was only one-half that given by the normal rats. Both extracts produced a high degree of follicle stimulation, luteinization of the membrana granulosa, and interstitial cell development, in the ovaries of the hypophysectomized rats (Fig. 4). The presence of the pituitary gland in the test animal does not seem to affect significantly the qualitative nature of the response of the ovaries to extracts of pregnant mare serum, although the amount of luteinization which is produced in the ovaries of the hypophysectomized rats is considerably less than that which was observed in the ovaries of the intact rats.

Comparison of the Apparent Interaction of Extracts and Antisera in Normal and Hypophysectomized Rats.

Extract of gelding pituitary and antiserum to extract of ox pituitary gland. It has recently been shown by Rowlands [1938 b and 1939] that the serum of rabbits, which have received a prolonged course of daily injections of extract of ox pituitary gland, has the power to neutralize selectively the luteinizing activity of an extract of gelding pituitary gland. In this way the extensive luteinization of the ovary of the immature rat, caused by the extract given alone, can be changed to pure follicle stimulation, with only a slight decrease in the response as measured by increase in ovarian weight. The evidence mentioned above that the pituitary gland of the test animal is able to secrete luteinizing hormone as a result of stimulation of the follicle in the ovary suggests that the amount of anti-luteinizing serum required to suppress luteinization of the ovary in the normal immature rat injected with extract of gelding pituitary gland, represents the amount required to neutralize, not only the luteinizing hormone contained in the extract itself, but also that secreted by the pituitary gland of the test animal. It seemed desirable, therefore, to determine the amount of antiserum required in the hypophysectomized rat to produce a similar effect, so that the amount of luteinizing hormone secreted by the pituitary gland of the test animal could be estimated. The results are given in Table IV and Text-fig. 1, p. 32.

In the normal immature rat it was found that 1·25 c.c. of Serum A com-

pletely inhibited the luteinizing action of 2·5 mg. of AP70B. With these amounts of serum and extract the ovaries, which weighed 46 mg., contained numerous large mature follicles which were devoid of luteinization.

Table IV. *The effect, on the ovaries and uteri of hypophysectomized rats, of various amounts of Serum A on a constant dose (2·5 mg.) of gelding pituitary extract (AP70B)*

Amount of serum c.c.	No. of rats	Average weights		Percentage of animals in which luteinization was inhibited
		Ovaries mg.	Uteri mg.	
0·0	8	52	64	0
0·02	4	49	98	0
0·03	6	29	70	33
0·06	8	25	67	25
0·10	7	31	71	86
0·25	6	24	83	83
0·50	4	18	64	100

Luteinization in the ovaries of the hypophysectomized rat was almost completely inhibited by 0·1 c.c. to 0·25 c.c. of the same serum. In each of the groups receiving these dosages, one rat showed slight traces of luteinization of the membrana granulosa, although the ovary as a whole showed pronounced follicle stimulation. Complete suppression of luteinization was effected with 0·5 c.c. of serum. It is shown, therefore, that the luteinizing activity of the extract in the hypophysectomized rat can be neutralized by about $\frac{1}{2}$ to $\frac{1}{5}$ the amount of serum required in the normal rat; a large part of the luteinization occurring under the conditions of the experiments reported by Rowlands (1938*b*) is accordingly due to luteinizing hormone secreted by the rat's own pituitary gland. This is in agreement with the results of Tyndale, *et al.* [1938].

The average weight of the ovaries in the three groups of hypophysectomized rats, receiving 0·1 c.c., 0·25 c.c., and 0·5 c.c. of serum and the standard dose of extract was 31, 24, and 18 mg. respectively, which is less than half that produced in the immature test rats showing an equal inhibition of luteinization. It must be remembered, however, that the control ovarian weight in the hypophysectomized rat is only about one-half that in normal rats of similar body-weight. If increase in ovarian weight is calculated as a percentage of initial weight, then the proportion of response compatible with complete absence of luteinization, under the conditions of the experiment, is much the same in the hypophysectomized as in the normal rats. In this connexion it may be noted that the dose/response curve [Rowlands, 1938 *b*], for AP70B given alone to normal immature rats, shows that an average ovary weight of nearly 30 mg. may be reached without all the animals in the group showing luteinization.

In the hypophysectomized rat, as shown in Table V, luteinization may not be general when an amount of the extract alone is injected sufficient to cause an increase of the weight of the ovary to an average of 29 mg.
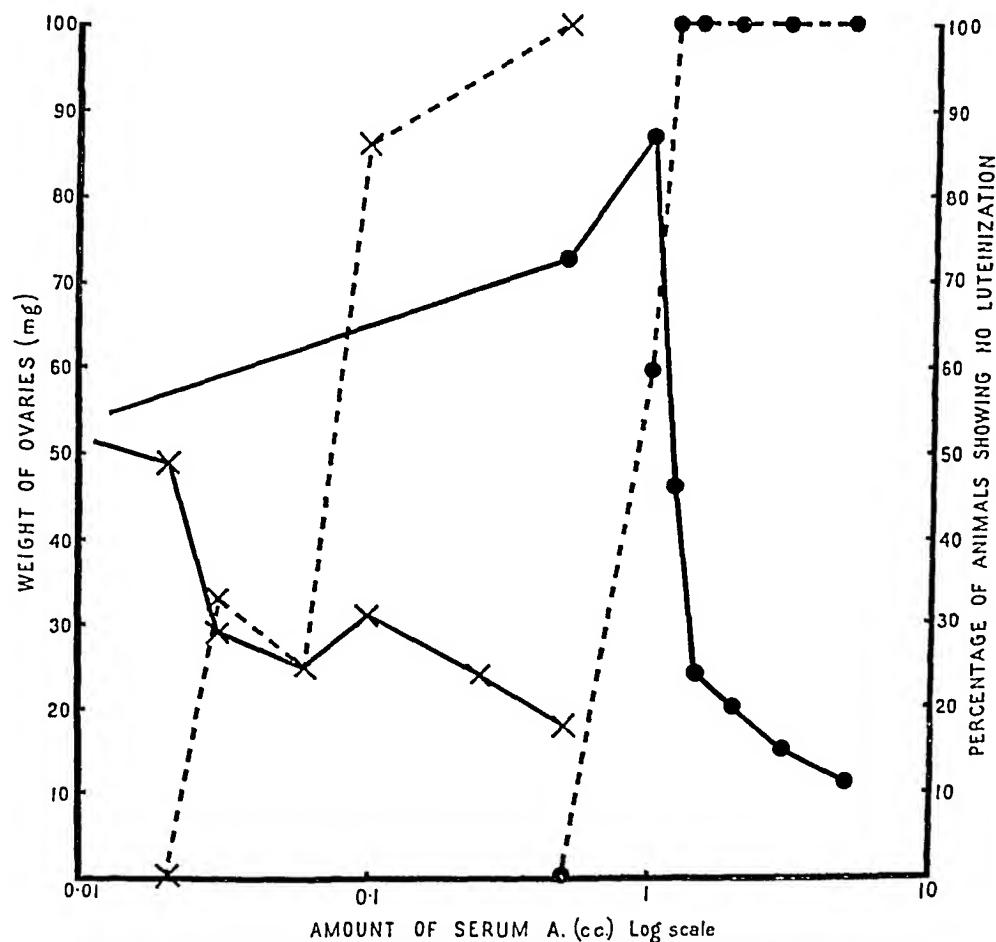

FIG. 1. The effect on the weight of the ovary and on the inhibition of luteinization, of varying amounts of Serum A on a constant amount of gelding pituitary extract in normal [Rowlands, 1938 a] and hypophysectomized rats.

●——● = weight of ovary of normal rat.
●----● = percentage inhibition of luteinization in normal rats.
×——× = weight of ovary of hypophysectomized rat.
×----× = percentage inhibition of luteinization in hypophysectomized rats.

To this extent the experiments on hypophysectomized rats, in comparison with those on intact rats, seem to provide less strong evidence of the selective neutralization of the luteinizing activity as opposed to mere quantitative neutralization of the extract as a whole. The serum, however, is clearly more effective in neutralizing the effect of the extract on hypophysectomized than on normal rats.

Fig. 1 above suggests another difference between the results obtained in

normal and hypophysectomized rats. In the former there is an augmentation of the effect of the standard dose of extract when small amounts of serum are given, but in hypophysectomized rats, this effect appears to be

Table V. *Assay of extract of gelding pituitary gland (AP70B) on hypophysectomized rats*

Amount of extract mg.	No. of rats	Average weights		Luteinization %
		Ovaries mg.	Uteri mg.	
1·00	4	7	15	0
1·75	7	29	50	60
2·50	6	55	61	100

absent. This observation may perhaps be instructive, but it cannot be dealt with here.

That anti-luteinizing serum is more effective in the hypophysectomized than in the normal rat has been confirmed with another antiserum (Serum B). The rate of development of anti-luteinizing activity in this serum, from rabbits chronically injected with extract of ox pituitary gland, has already been determined using normal rats as test animals [Rowlands, 1939]; similar tests on samples of serum taken at four different stages during immunization have now been carried out in hypophysectomized rats.

Table VI. *Comparative activity, in normal and hypophysectomized rats, of varying amounts of Serum B and a constant amount of 2·5 mg. of gelding pituitary extract (AP70B)*

Period over which rabbits, yielding Serum B, were injected Weeks	Assay in hypophysectomized rats				Amount of Serum B required to produce same degree of inhibition of luteinization in normal rats c.c.	Activity of Serum B in hypophysectomized rats compared with that in normal rats
	Amount of Serum B injected c.c.	Weight of ovaries mg.	Weight of uteri mg.	Inhibition of luteinization %		
4½	0·05	40	58	50	0·25	5 : 1
8½	0·05	29	100	83	0·20	4 : 1
9½	0·02	26	68	83	0·10	5 : 1
12½	0·02	29	129	83	0·05	5 : 2

Calculation of the approximate amount of serum required to cause an equal degree of inhibition of luteinization in normal rats (Table VI) shows that Serum B is from 2½–5 times as effective in producing this inhibitory effect in hypophysectomized rats.

Extract of urine of pregnancy and antiserum to extract of ox pituitary gland. It has previously been shown [Rowlands, 1937] that an antiserum to an extract of ox pituitary gland inhibits to some extent the quantitative response of the ovaries of normal rats to an extract of urine of pregnancy. Complete neutralization of the effect of the extract, however, was not

obtained even with comparatively large amounts of antiserum. This test has been repeated, using hypophysectomized rats. Since there is only a very slight quantitative response in the ovaries of the hypophysectomized rat to extracts of urine of pregnancy, it is necessary to base the result on the histological changes of the ovary.

The description of the histological changes in the ovaries of the hypophysectomized rats stimulated with extract of urine of pregnancy (UP. 10), which serve as controls is given on p. 28. All the elements of the ovary of an animal so treated tend to become uniformly luteinized. The injection of 1 c.c. and 2·5 c.c. of an antiserum (Serum A) [Rowlands, 1938 b], obtained in rabbits by the injection of an extract of ox pituitary gland, failed to inhibit the luteinization which occurs in response to extracts of urine of pregnancy. It is likely, therefore, that the inhibition of the effect of this extract which was previously obtained in intact rats is caused by the neutralization of the endogenous gonadotrophic hormones of the rat, rather than by the direct action of the antiserum on the gonadotrophic hormone contained in the extract of human urine of pregnancy which is injected. The results are therefore comparable with those of Collip, Selye, and Williamson [1938], who found that the ovaries of rats previously rendered insensitive to pig pituitary gland responded subsequently to human urine of pregnancy extract in a manner similar to that in the hypophysectomized rat.

SUMMARY

1. The comparative effects of various gonadotrophic preparations have been observed on the ovaries of intact and hypophysectomized immature rats. The extracts were prepared from the pituitary glands of various species including man, horse, sheep, pig, and ox, and also from the urine and serum of pregnant women, the urine of a senile and of an ovariectomized woman, and the serum of pregnant mares.

2. Extracts of the urine and serum of the pregnant woman and of ox pituitary gland cause only generalized luteinization of the ovaries of hypophysectomized immature rats, but cause follicular growth as well as luteinization in intact immature rats. The difference in the response may be attributed partly to the greater initial size of the follicles in the intact rats, but is probably largely due to stimulation of the pituitary gland of the intact rat when luteinization of the ovary begins.

3. Preparations of certain human pituitary glands cause only follicular growth in the hypophysectomized rats, whereas in the intact rats they produce follicular stimulation and luteinization. This difference must be ascribed to a normal or an evoked luteinizing activity by the pituitary gland of the intact immature rat.

Fig. 1. Ovary of rat (HRt 150) 10 days after hypophysectomy at 40 to 50 g. body-weight. Most of the follicles are in an atrophic condition. × 16

Fig. 2. Ovary of hypophysectomized rat (HRt 84), injected with an extract from the serum of pregnant woman (PWS 77), showing generalized luteinization. × 16
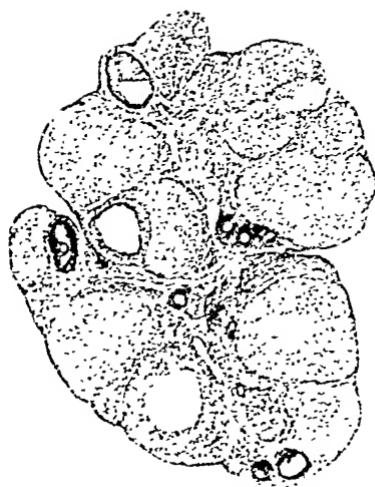

Fig. 3. Ovary of incompletely hypophysectomized rat (HRt 85), injected with a similar amount of extract PWS 77, showing development of corpora lutea and luteinization of membrana granulosa. × 16
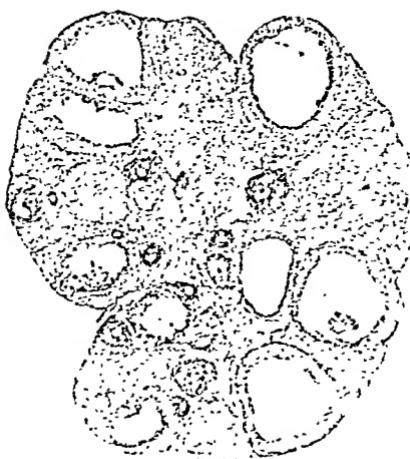

Fig. 4. Ovary of hypophysectomized rat (HRt 496) injected with extract from serum of pregnant mares (PMS 18), showing extensive follicle stimulation accompanied by luteinization of membrana granulosa. × 16
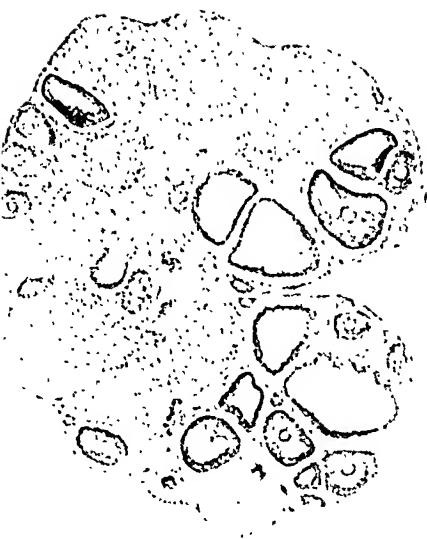
Fig. 5. Ovary of hypophysectomized rat (HRt ..., injected with extract of gelding pituitary ..., showing numerous large corpora lutea ...nd several large normal follicles. × 16

Fig. 6. Ovary of hypophysectomized rat (HR 480), injected with extract of pig pituitary gland showing small corpora lutea atretica arising from heavily luteinized follicles. × 16
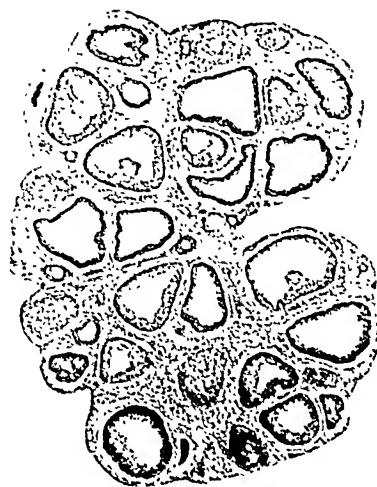

Fig. 7. Ovary of hypophysectomized rat (HRt 687), injected with extract of the pituitary gland (HP 737) of a woman aged 57 years, showing numerous large follicles without any luteinization. × 16
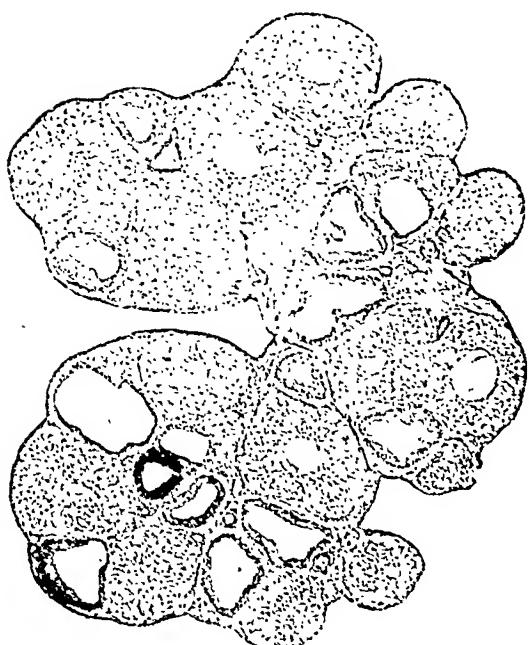
Fig. 8. Ovary of intact rat (R 7481), treated with a similar amount of human pituitary (HP 737), showing heavily luteinized follicles forming large corpora lutea atretica. × 16

4. Extracts of horse, sheep, and pig pituitary glands, and of the serum of pregnant mares stimulate the growth of follicles and the production of luteinization in both intact and hypophysectomized rats.

5. The differential neutralization of the luteinizing activity of an extract of gelding pituitary gland, by antisera to an extract of ox pituitary gland, can be effected with much smaller amounts of antiserum in the hypophysectomized rat than in the normal rat. This difference is almost certainly due to the production of luteinizing hormone by the pituitary gland of the intact test animal, stimulated by the follicular growth in the ovary.

6. Rabbit antiserum to ox pituitary gland has no neutralizing action on the effect of an extract of human urine of pregnancy on the ovaries of the hypophysectomized rat. The strong, though incomplete, neutralizing action of the same antiserum, on the effects of this same urinary extract on normal rats must be due to its action on the hormones of the rat's own pituitary gland, and not on the gonadotrophic substance of the human urine.

7. Earlier conclusions with regard to the specificity of antisera to pituitary extracts and analogous principles may need revision in the light of these findings.

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DEPRESSION OF HYPOPHYSEAL ACTIVITY BY THE IMPLANTATION OF TABLETS OF OESTRONE AND OESTRADIOL

By RUTH DEANESLY

From the National Institute for Medical Research, London, N.W. 3

INTRODUCTION

NUMEROUS workers have reported that continued administration of oestrogenic preparations by daily subcutaneous injections will damage the reproductive organs of male rats. At first the damage was attributed to hormone antagonism [Steinach and Kun, 1926; Laqueur and de Jongh, 1928; Borchardt, Dingemanse, de Jongh, and Laqueur, 1929], but later when it had been shown that similar results could be obtained in female rats and mice[1] it became likely that the gonadotrophic activity of the pituitary gland was affected. Moore and Price [1930, 1932], who reviewed the older papers, showed that the harmful effect of oestrogenic extracts in the male rat could be prevented by simultaneous injection of gonadotrophic substances which replaced the secretion of the animal's own pituitary. These writers found severe changes in the normal male rat after 20 days injection with 15–20 rat units of oestrogen daily—a comparatively low dose. Spencer, Gustavson, and D'Amour [1931] first showed that oestrogen injections into immature rats not only affected the reproductive organs but also arrested growth. Wade and Doisy [1931] obtained similar results. In various experiments Spencer and his collaborators [1932a and b] studied the effect of oestrogen alone or combined with a gonadotrophic extract on normal male rats and on normal and ovariectomized females. They confirmed Moore's findings and made histological studies of the gonads, accessory organs, and endocrine glands. The work was extended by Halpern and D'Amour [1934, 1936], who found that 805 rat units of oestrogen in 8 weeks caused degeneration of the male gonads comparable to that seen after hypophysectomy. McEuen [1936] obtained similar results. These writers described the enlargement of the rat hypophysis which occurs after oestrin injections; this has been found in mice and rats of both sexes, by numerous other workers including Hohlweg [1934], Korenchevsky and Dennison [1934], Nelson [1934], Wolfe and Phelps [1935], Cramer and Horning [1936a], and McEuen, Selye, and Collip [1936] after administra-

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tion of oestrogenic substances. In rats so treated for 7–11 months tumours of the anterior pituitary gland weighing up to 250 mg. may be formed [Zondek, 1936*b*, 1937; McEuen *et al.*, 1936; Wolfe and Wright, 1938]. A low gonadotrophic hormone content has been found in the hypophysis of both male and female gonadectomized rats [Meyer, Leonard, Hisaw, and Martin, 1930, 1932; Bialet-Laprida, 1933] and normal male rats [Lipschütz, 1935] after continued injections with oestrogen.

Zondek [1936, 1937], extending his earlier work on the subject, used larger doses of oestrogen for longer periods and showed that it was possible to depress the growth-rate of both rats and birds (White Leghorns) by regular injections. He states that the injections acting on the hypophysis cause first a failure in the secretion of the gonadotrophic hormone and then shrinkage of the gonads and accessory organs. The growth-rate is next affected, but the other pituitary gland functions do not seem to be impaired, although the pituitary gland and adrenals are enlarged in male rats. Bialet-Laprida [1933], however, found thyroid depression in the rat, and Emmens [1938] in the fowl, after oestrogen administration, and Barnes, Regan, and Nelson [1933] claim to have induced depression of the diabetogenic hormone of the anterior pituitary by similar means.

Noble [1938*a* and *b*] has shown that the inhibition of the anterior pituitary gland in rats, with the accompanying changes in the reproductive organs, can be brought about by synthetic oestrogens.

The effects of prolonged administration of oestrogenic substances have also been studied on male mice, mainly on cancer-susceptible strains. In spite of negative results such as those of Allanson [1931] it is clear from the findings that the gonadotrophic function of the pituitary of the mouse, like that of the rat, may be inhibited by oestrogenic substances [Lacassagne, 1934; Burrows, 1936*a*; Cramer and Horning, 1936*b*; Gardner, 1937]. The histological changes found in the various organs will be briefly referred to later in this paper. A special study has been made of mouse adrenals by several workers, including Martin [1930], Burrows [1937], Lacassagne and Raynaud [1937], Cramer and Horning [1936*b*, 1937], and Danner [1938], but the results have been inconsistent, probably because of differences in technique.

Although the literature on oestrogen administration is extensive, there are few reports about the recovery of the rats and mice after cessation of the treatment. Spencer *et al.* [1932*a*], however, investigated this point on three male and five female rats and showed clearly that after the end of treatment an increase in the growth-rate takes place and the reproductive organs tend to return to functional normality. The females show complete restoration and a return of fertility more rapidly than the males. Only one out of three males is reported to have contained live sperm 12 weeks after

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cessation of the injections. Zondek [1936b] states that, if injections are discontinued after several months, the animals do not spontaneously resume their growth.

It has recently been demonstrated [Deanesly and Parkes, 1937, 1938; Noble, 1938a; Pybus and Miller, 1938] that the effect of prolonged administration of oestrogens can be obtained in birds, rats, and mice by placing a compressed tablet of pure hormone in the subcutaneous tissues. Such tablets will last for a very long period [Deanesly and Parkes, 1938] owing to their low rate of absorption, and the necessity for continued daily injections is thus avoided. In the present paper a further account is given of the results of administration of oestrogens in tablet form to male rats and mice, with new data on the rate of absorption of oestrone and oestradiol, on the changes in size and structure in the reproductive organs and ductless glands, and on the gradual recovery of the animals after removal of the tablets.

Material and Technique

Oestrone was used in the form of circular, generally disk-shaped, tablets weighing 2·25–16 mg. Three large tablets weighing 80–96 mg. were also used. The oestradiol tablets were similar and weighed 11·8–16·5 mg.

The mice and rats were normal males of varying weights. Subcutaneous implantation of the tablets was carried out aseptically under ether anaesthesia, and the skin was closed by a stitch. Later removal of tablets was carried out under the same conditions. The tablets were weighed, generally to the nearest tenth of a milligram, before implantation, and cleaned, dried in a desiccator, and reweighed after removal.

The Rates of Absorption of Oestrone and Oestradiol in Tablet Form

It was not always possible to make an accurate determination of the loss of material from small tablets during short periods of implantation, since the estimate of the slight amount absorbed might be disturbed by adherence of traces of tissue, or by the loss of small amounts of the substance during dissection of the tablet from the subcutaneous tissues. Over longer periods of implantation the errors of this nature are relatively smaller, and consistent results were obtained.

There is as yet no good evidence that the rate of absorption of the tablets is affected by the functional requirements of the animal; factors influencing the rate of absorption are discussed in an earlier paper [Deanesly and Parkes, 1938]; the figures in Table I provide further data, based on more animals. The last column of the table shows, for convenient comparison, the percentage loss from the tablets per month (30 days). For oestrone in mice this figure is rather lower than that given by Deanesly and Parkes [1938], but for oestrone and oestradiol in rats the figures are

substantially similar. It will be seen that the absorption of oestrone is very gradual and, assuming the animal's survival, a tablet of 15 mg. would probably persist subcutaneously for 18 months or 2 years. At the time of writing [February, 1939] some rats have carried tablets which are still palpable after 9 months. It is not known why the rate of absorption is higher in mice than in rats, though it may be due to a difference in body temperature or metabolism. Pybus and Miller's [1938] estimate of the absorption from 5 mg. oestrone tablets in mice is almost certainly too high. It is clear that the absorption of oestradiol under the skin of rats is more than twice as rapid as that of oestrone—a 14 mg. tablet loses 8% or 9% per month as compared with 4% per month or less for a similar sized tablet of oestrone.

The data for oestrone and oestradiol implanted for 263 and 270 days (Table I) show that the rate of absorption, expressed as a percentage of the original tablet, falls as the duration of the experiment is prolonged. This result would be expected owing to the steady decrease and reduced surface area of the original tablet. Further studies are now being made of absorption over long periods. At any stage of an experiment the tablets can be removed from the subcutaneous tissues (under ether anaesthesia), dried, weighed, and replaced the following day. This was done for the tablets in groups I, II, and IV (Fig. 1).

Table I. *Absorption of oestrone and oestradiol from the subcutaneous tissue of mice and rats*

Substance	Species group no.*		No. of animals	Average tablet size mg.	Days implanted	Average amount absorbed mg.	Approximate % absorbed per month (30 days)
Oestrone	Mice		11	9·00	50	0·5	5·6
"	Rats		5	14·73	75	1·63	4·4
"	"		1	15·40	112	1·7	3·0
"	"	I	4*	16·90	127	2·3	3·2
"	"	II	4*	6·85	127	1·16	4·0
"	"		2	15·90	150	2·1	2·65
"	"		1	16·00	263	4·1	2·9
Oestradiol	"	III	5*	14·40	50	2·1	8·75
"	"		4	14·30	60	2·5	9·00
"	"	IV	5*	11·80	121	4·2	8·80
"	"		2	16·25	150	5·05	6·25
"	"		3	14·50	270	7·3	5·60

* Fig. 1 shows the growth-rates of these groups of rats. Group II contained five rats, but satisfactory weight determinations could only be made in four tablets.

THE EFFECTS OF OESTROGENIC SUBSTANCES IN MALE RATS

Growth. As stated by Halpern and D'Amour [1932], adult male rats (such as most of those listed in Table II) show little change in weight after administration of oestrogens. Male rats which have not reached adult size,

however, show a marked reaction to oestrogen administration in their growth-rate. Fig. 1 shows the average growth-curves of 35 normal rats[1] and of 20 other rats (groups I to IV, Table I) kept under identical conditions but having subcutaneous tablets of oestrone or oestradiol. The tablets were implanted on 18 July, when the rats at 120 g. body-weight


FIG. 1. Average growth-curves of male rats.

●——●——● 35 normal rats. The remaining 4 lines represent the growth-curves of rats in which tablets of oestrogen were implanted on 18 July (arrow A).

×— –×– –× Group I. 5 rats, 4 after 3 Sept., receiving oestrone tablets, average weight 16·9 mg.

■——■——■ Group II. 5 rats receiving oestrone tablets, average weight 6·9 mg.
The slight divergence between the growth-curves of groups I and II only occurs after the death of a rat in group I and is probably not significant.

●---●---● Group III. 5 rats receiving oestradiol tablets, average weight 14·4 mg.; on 6 Sept. (arrow B) the tablets were removed.

●– –●– –● Group IV. 5 rats receiving oestradiol tablets, average weight 11·8 mg.

were about half grown. The graphs of the rats with tablets, with the exception of group I, each represent an average of five animals.

It will be seen that the rate of growth falls off sharply very soon after the implantation of oestrogenic tablets, although the animals continue to gain weight slightly. From the data in Table I it may be calculated that the average daily amount absorbed is 18 μg. and 8 μg. of oestrone in groups I and II, and 42 μg. and 35 μg. of oestradiol in groups III and IV. These amounts are comparatively low and of the same order as those injected by Spencer *et al.* [1932*b*]. Even the relatively low dose of oestrone absorbed

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[1] During the last week of September 1938 the rats show a loss of weight apparently due to disturbance of their maintenance routine.

by group II was sufficient to inhibit growth to about the same extent as in the other groups. The graph of group III shows the effect of removal of the oestradiol tablets after 50 days' implantation. An immediate increase in the growth-rate takes place, as was found by Spencer *et al.* [1932*a*], after the cessation of injections, and in a few weeks the animals reach a normal body-weight. Group III was killed on 28 October for examination of the reproductive and endocrine organs, 52 days after removal of the oestradiol.

Further experiments are now in progress to ascertain the effect of different amounts of oestrogenic substances, acting through the hypophysis, on the growth of younger rats.

Changes in the size and structure of the reproductive organs and ductless glands. The effects of single implantations of oestrogens in tablet form on the pituitary glands, adrenals, and reproductive organs of male rats are illustrated in Table II, which shows the rapid decline in the size of the reproductive organs and the recovery after removal of the tablet.

Table II. *Size changes in the reproductive organs, pituitary gland, and adrenals of the male rat after implantation of oestrogens in tablet form*

No. of rats	Sub-stance	Days implanted	Days after tablet removal	Testes g.	Prostate mg.	S.V. mg.	Pituitary gland mg.	Adrenals mg.
4	Control*	—	—	2·30	597	610	8	27
4	Oestrone	28–30	—	0·95	196	82	16	52
1	Oestradiol	30	—	0·68	320	127	24	29
4	Oestradiol	60	—	0·45	103	55	37	43
6	Oestrone	103–11	—	0·45	106	57	21	45
2	Oestrone	150	—	0·31	105	50	—	42
1	Oestrone	263	—	0·44	160	61	30	40
2	Oestradiol	150	—	0·27	148	73	39	38
3	Oestradiol	270	—	0·29	87	86	Tumours	32
5	Oestradiol	50	52	1·86	487	365	13	39
4	Oestrone	111	47–50	2·77	552	522	10	34

Column header note: "Average weight of" spans Testes, Prostate, S.V., Pituitary gland, and Adrenals.

* The reproductive organs and pituitary glands are from normal rats average weight 196 g., the adrenals from slightly younger rats averaging 181 g.

Pituitary gland. Enlargement of the rat pituitary gland is noticeable 15 days after implantation of the oestrogenic substance, and the process commonly continues until the gland has increased from about 8 mg. in an adult rat to 22 mg. Exceptionally, particularly in male rats with oestradiol tablets for 60 days, the gland may reach a weight of over 50 mg.

Histological examination of the anterior lobes of the pituitary glands from the present series of rats confirmed the accounts given by previous workers of the cytological changes accompanying the enlargement [Severinghaus, 1937]. The early response consists of a degranulation of

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Histological examination of the anterior lobes of the pituitary glands from the present series of rats confirmed the accounts given by previous workers of the cytological changes accompanying the enlargement [Severinghaus, 1937]. The early response consists of a degranulation of

some of the basophil cells; later they enlarge and their Golgi apparatus hypertrophies. With continued oestrogen treatment there is widespread degranulation and enlargement not only of the basophils but also of the acidophils; the chromophobe cells increase in relative number owing to the degranulation and also absolutely, owing to mitotic division.

After prolonged oestrogen administration the pituitary gland has a uniform chromophobe appearance, the cells being swollen and having distinct cell boundaries and no granules. Part of the enlargement is due to marked vascular congestion. In the anterior lobe there is much colloid accumulation and large areas of degenerating cells. In some cases the centre of the lateral areas of the anterior lobe consists of extravasated blood and degenerating cells.

In spite of the extensive changes caused by continued oestrogen administration, the pituitary glands recover their normal histological appearance within 50 days of the removal of the tablets. Such glands have regressed towards a normal size (Table II), and sections show granular basophil and acidophil cells and normal capillaries.

Haemorrhagic pituitary tumours, apparently similar to those described by earlier workers, were found in only three rats—those which had oestradiol tablets implanted for 270 days. Two of the tumours weighed 236 mg. and 356 mg. respectively when fresh. The rats were not obviously affected by the tumours, but appeared to be in a bad condition when killed. It will be noticed that the weights of their reproductive organs and adrenal glands are similar to those of other oestrogen-treated animals. Although Zondek [1937] states that in his experience the duration of the oestrogen treatment, rather than the dosage, determines tumour formation, yet the rat absorbing oestrone over a similar period (263 days) showed no signs of tumour formation.

Testes and accessory glands. Table II gives a general indication of the fall in the weight of the testes, prostate, and seminal vesicles of adult male rats after implantation of oestrone or oestradiol tablets. The actual size of these organs at any given stage of the experiment depends partly on the initial body-weight of the animal. Most of the rats listed in Table II weighed over 170 g. when tablets were implanted, but a few were young adults, 140–50 g. in weight. The shrinkage of the accessory glands is more rapid than that of the testes, a fact that can be correlated with the degenerative changes in the interstitial cells. These are apparent as early as 14 days after tablet implantation; the nuclei shrink, the cells become vacuolated and lose their cell boundaries, and the spaces between tubules normally occupied by interstitial cells become filled with eosinophil colloid. Both prostate and seminal vesicles show marked decrease in size due to loss of secretion after 14 days, and the maximum amount of shrink-

age has generally occurred after 30 days. Although oestrone is known to have a direct action on rudimentary or atrophic rat seminal vesicles, [Freud, 1933] where it stimulates fibrosis and causes a slight enlargement, it is doubtful if it has a significant effect on size in this series. The testes vary greatly in weight (0·59–2·8 g.) after about a month, but all show degenerative changes in the interstitial cells and disorganization of the germinal epithelium in many of the tubules, although spermatozoa may still be present in others. Rats from the same group, 30 or 38 days after implantation, may differ markedly in the size and condition of the testes, although the prostate and seminal vesicles were equally shrunken.

The condition of the testes and accessory organs tends to confirm the finding of Halpern and D'Amour [1936] that males treated with oestrogen soon become infertile—in some cases after 19 days. In a group of rats treated for 50 or 60 days the testes were very degenerate and comparable to those of hypophysectomized animals.

These degenerative changes in the gonads and accessory organs are reversible, as indicated by the weights of the organs from the last groups of rats listed in Table II. The tablets were removed from these animals when the testes had obviously regressed, and the rats were killed about 50 days later. At the time of death the testes had increased in size and were no longer flabby, and the prostate and seminal vesicles were well developed. Although the histological appearance of the interstitial cells of the testis was variable, the condition of the accessory glands left no doubt as to their functional activity. Sections showed that in about half the testes the germinal epithelium was not fully restored, although some tubules usually contained spermatozoa. One of the rats in the last group mated with a normal female 39 days after removal of the tablet, but the mating proved infertile. The other rats in this group did not mate, although placed with normal females. On the whole the rats recovering after oestrone showed a more normal germinal epithelium than the rats which had received oestradiol, although the implantation period was shorter in the latter (Table II). It seems probable that the degree of recovery depends both on the amount of oestrogenic substance absorbed and on the age of the rats when the tablets are implanted and removed. Fifty days after tablet removal, when the accessory glands are again well grown, spermatogenesis is generally re-established and spermatozoa are found in the epididymis.

Adrenals. It has long been known that the adrenals are smaller in the male rat than in the female. Winter and Emery [1936] show that gonadectomy increases adrenal size in the male rat and decreases it in the female. Korenchevsky and Dennison [1934, 1935] find that administration of oestrogens causes an increase in adrenal weight in the male rat but not

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There was a tendency for the former to resemble the adrenals of females in size, shape, and histological structure. The cortex became thicker and more rounded, and a zone of small cells, generally with deeply staining cytoplasm, developed next the medulla. These changes have been illustrated by Burrows [1936b], who compares the condition in the adrenal of the male after oestrogen dosage with that found in the adrenal of the castrate. It is well known that castration causes the development of an inner cortical zone (the so-called X-zone) in the male adrenal, which resembles that found in the pre-pubertal female. Typically the X-zone has well-defined limits, and in this respect it differs from the zona reticularis which replaces it in the adult female. The zone which develops round the medulla, as the male adrenal enlarges during oestrone treatment, resembles more closely the somewhat indefinite zona reticularis of the older female than it does the X-zone. It is subject to gradual degeneration and change, the cytoplasm sometimes tending to lose its affinity for stains so that the zone becomes less distinct from the rest of the cortex. In some of the experimental males the adrenals were of the female type with a large cortex, but the zona reticularis or X-zone had disappeared, though there were cells showing fatty degeneration next the medulla.

After removal of the oestrone tablets the adrenals gradually reverted to the male size, shape and appearance.

Mammary glands. Gardner, Smith, and Strong [1935], Burrows [1936a], and Nelson [1936] have described the effect of oestrogens on the mammary gland rudiment in male mice. No detailed observations were made on it in the present relatively short experiments, but slight proliferation of the ducts was observed in some mice.

SUMMARY

The effects of oestrone and oestradiol have been investigated by the implantation of compressed tablets of pure hormone under the skin of male rats and mice for periods from 10–270 days. This treatment, like continued injections of oestrogens, causes inhibition of some of the functions of the pituitary gland. It leads to marked depression of the growth rate, loss of fertility, shrinkage of gonads and accessory organs, and enlargement of the adrenals and pituitary gland. After removal of the oestrone or oestradiol tablets these changes are reversible, and a study has been made of the gradual recovery of the animals. Three rats with oestradiol tablets for 270 days developed tumours of the pituitary gland, which would probably eventually have proved fatal.

Records are included of the rate of absorption of the tablets.

rudimentary glands, due to fibrosis, is only slight and not comparable to that produced by interstitial cell activity or androgenic hormones. After 50 days' oestrone treatment the seminal vesicles of immature mice weighed only 10–20 mg., including the cranial prostate lobes. Table III shows that in adult mice there is marked shrinkage of the seminal vesicles in the first fortnight of oestrone treatment, suggesting that the interstitial cells are rapidly inactivated. Burrows [1935, 1936a] and Gardner [1937] have described and illustrated histological changes in these cells after treatment with various oestrogens; both these workers used immature male mice where the volume of interstitial cell tissue is higher in relation to that of the tubules than in adults. They describe a degenerative process of cell enlargement, associated with subnormal male hormone production. A similar process has been seen in the present experimental series in immature mice which have received oestrone for 59 days. The interstitial cells are large and lipoid-containing with distinct cell limits, but the nuclei appear shrunken and degenerate. Over shorter periods, when less shrinkage of the tubules has occurred, the morphological changes are less distinct. In adult mice receiving oestrone the interstitial cells also show degenerative changes, but the enlargement phase is less common or of shorter duration; the interstitial cells often appear shrunken and surrounded by eosinophil colloid.

Adrenals. In the mouse the adrenals are small and often somewhat elongated in the male, and larger and more rounded in the female, a sex difference which has been widely studied [see references and discussion by Deanesly, 1938]. There is evidence that the growth of the male adrenal cortex is inhibited by the secretion of the testis, since castration leads to cortical enlargement. As regards the effect of oestrogens on the male mouse, a study of the literature indicates that the different experimental results on the adrenal cortex can be roughly classified into three groups: (1) no change after short periods of injection [Martin, 1930]; (2) cortical enlargement and somewhat variable histological changes after longer periods of treatment [Martin, 1930; Burrows, 1936b]; and (3) marked degenerative changes in the cortex and fatty accumulations round the medulla after prolonged oestrogen treatment [Lacassagne and Raynaud, 1937; Cramer and Horning, 1937; Danner, 1938]. The present results fall into the second category, since the adrenals show definite enlargement and histological changes during oestrone treatment. As in the rat, this can be regarded either as a response to the direct 'feminizing' influence of oestrone, or as a response to the inhibition of the testis caused by oestrone acting on the pituitary gland—in other words, as a castration effect.

The adrenals were examined from 10 mice, immatures and adults, during oestrone treatment, and from six others after its discontinuance.

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AN ASSAY METHOD FOR PROGESTERONE BASED UPON THE DECIDUAL REACTION IN THE RAT[1]

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STUDIES on the physiology of progesterone in the rat have met with difficulties because of the lack of clear-cut anatomic end-points of progestational effects. Detailed studies have heretofore been centred on the rabbit, wherein the transformation of the endometrium and the quiescence of the uterine musculature present striking evidence of corpus luteum hormone action. In the rat, uterine changes during pseudopregnancy, lactation, and after progesterone injections are by no means as striking. The decidual reaction, on the other hand, is a definite and specific indicator of corpus luteum activity in the rat and has therefore yielded the most exact information regarding the endocrine relations of early pregnancy. Because of its high degree of specificity, this response has been adapted to the assay of progesterone. Rats are more readily available in many laboratories than rabbits, and a satisfactory assay method based upon the rat may be useful in certain instances in preference to the more expensive rabbit assay.

Weichert [1928], by the use of corpus luteum extracts, was the first to produce deciduomata in ovariectomized rats. This effect of crude extracts was confirmed by Nelson and Pfiffner [1930] on adult, and by Shelesnyak [1933] on immature, animals. The latter author obtained positive responses with as little as 4 g. equivalent of fresh corpus luteum tissue as the total dose over 8 days, while Nelson and Pfiffner gave 8 to 10 g. equivalents daily. The production of deciduomata in ovariectomized rats by means of progesterone has been reported in a preliminary communication [Astwood, 1939]. Experiments on ovariectomized rats showed that it was difficult to obtain deciduomata when such animals were first primed with oestrogen and then treated with large amounts of progesterone with or without various dosages of oestrogen. In the majority of such animals, endometrial trauma on the 3rd to the 5th day of progesterone treatment resulted in no decidual reaction with progesterone dosages as high as 2 mg. daily. Similarly, various fractions from fresh hog corpora lutea of

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different degrees of purity resulted in inconsistent responses even when as much as 75 g. equivalent of fresh tissue were given daily. The methods of the previously mentioned authors were followed closely, but it was impossible to achieve the degree of successes described by them, though much larger dosages were employed. It was found, however, that progesterone is fully active in promoting the growth of deciduomata in the absence of the ovaries once the endometrium has been rendered sensitive to trauma by the normally functioning ovaries of the pseudopregnant animal. As a consequence of this, a procedure has been developed permitting the consistent formation of deciduomata in rats treated with progesterone. The degree of response obtained is proportional to the dosage given within certain limits.

Methods

Adult female rats, 3–5 months old, weighing 150–200 g., were used in these studies. Groups of 5 to 15 animals showing fully cornified vaginal smears were selected from the colony on the 1st day of an experiment. The cervix uteri of each rat was stimulated by a strong faradic current according to the method of Greep and Hisaw [1938]. Vaginal smears were then made once daily until the termination of the experiment. Four days after the cervical stimulus was applied, the animals were oöphorectomized by the lumbar route. At the same time, the endometrium of one uterus of each animal was traumatized by inserting a needle through the cut tubal end of the uterus as far as the cervix on that side. By withdrawing the needle slantwise, the antimesometrial aspect of the endometrium was scratched throughout its entire length by the needle point. The injections of progesterone were given for 3 days beginning immediately after operation, during which time the vaginal orifice was frequently inspected for external haemorrhage. The animals were killed with chloroform and the traumatized uterus examined for gross swelling as compared with the undamaged uterus of the other side. In some cases laparotomy was performed at 3 days and injections continued for 1 or 2 days longer to see whether further growth or regression would occur. The degree of gross swelling was rated from 1 plus to 4 plus, by comparison with deciduomata produced in uninjected pseudopregnant animals whose ovaries were left intact. A 4 plus rating was given those uteri showing a decidual response equal in diameter to those normally found at 3 days; 3 plus = normal 2-day deciduomata; 2 plus = normal 1-day deciduomata; and 1 plus = a questionable response. In some cases microscopic preparations were made to confirm the diagnosis, but it has been found that unless typical swellings are seen grossly, histologic effects are also questionable.

Control Procedures

As a measure of the efficiency of the cervical stimulus in inducing pseudopregnancy, reference may be made to a number of animals in which this procedure was performed and the animals then followed without oöphorectomy. The first 65 individuals became pseudopregnant from a single stimulus before a refractory animal was encountered. This exceptional animal and one other subsequently found could not be rendered pseudopregnant either by this means or by copulation. For 5 and 6 consecutive cycles respectively these animals, when caged with males, showed vaginal plus at each 4- to 5-day cycle without becoming pseudopregnant. Autopsy showed no gross abnormality.

Being relatively certain that the cervical stimulus used was effective, the next experiments were directed to the production of deciduomata in normal pseudopregnant rats. The endometrial traumatization was found to be most effective when performed 4 days after cervical stimulation during oestrus, that is, 5 days counting from prooestrus. The method used for inflicting adequate endometrial damage without operation consisted of exposing the cervix uteri with an aural-speculum and inserting a blunt steel wire possessing a small sharpened spur near the tip into the uterus on one side. The wire was run in until it was stopped by the tubal sphincter and then withdrawn. In all of 35 control animals treated in this way well-formed deciduomata developed in every instance.

That no decidual reaction takes place when the ovaries are removed at the time of endometrial trauma is shown by a group of 22 animals. These animals were treated in the same manner as the test animals with the exception that no injections were made, and were killed at various intervals from 1 to 4 days after oöphorectomy. In no instance did the traumatized uterus display any swelling that could have been rated even as a questionable response.

RESULTS

The degree of decidual reaction in response to various forms of treatment is set forth in the table. In the first place, it will be noted that a daily dose of 0·5 mg. or more of progesterone given alone invariably resulted in a 3 to 4 plus response, while 0·25 mg. progesterone gave in most instances a 2 plus reaction. Less than 0·25 mg. resulted in more variable responses, and 0·1 mg. daily gave as a rule only 1 plus reactions.

The influence of various amounts of simultaneously administered oestrogen can also be judged from the data in the table. A daily dosage of 0·1μg. oestradiol invariably caused inhibition of the response, and with the lower dosages of progesterone this inhibition was complete and no deciduomata developed. Dosages of 0·02μg. to 0·075μg. did not cause

inhibition, but likewise did not appreciably enhance the progesterone effect. These dosages of oestradiol prevented to some degree the atrophy which occurred in the non-traumatized uteri, and in isolated instances appeared to cause some augmentation of the progesterone. Taken as a whole, however, the results fail to show any dosage combination of oestrogen and progesterone which is more effective than is progesterone given alone.

Subdivision of the daily dosage into two or three injections gave no evidence that repeated injections over each 24-hour period are more efficient than single daily doses. Variation in the volume of injected oil from 0·1 c.c. to 1·0 c.c. at each dose likewise proved to have no demonstrable influence on the response. As suggested by Phillips and Young [1938], palmitic acid was tried to see if it had any influence on potentiating the action of progesterone. Small amounts of this substance, admixed with the sesame oil before injection, was without influence on the reaction. When the dosage of progesterone was dissolved in a large volume of 20% palmitic acid in sesame oil, the response was definitely inhibited. Testosterone and testosterone propionate, in doses of 1 to 10 mg. daily, were not effective in inducing decuoma formation under those conditions.

In the use of this reaction for the assay of progesterone, a 2 plus decidual response has been taken as the optimum reaction. It constitutes a definite enlargement of the traumatized uterus which cannot be mistaken for a questionable or negative response, and yet falls far short of a fully developed reaction. Three days has been taken as a standard time, for with the smaller doses no further growth occurs when injections are continued for 4 or 5 days, and sometimes regression takes place in spite of continued treatment. The amount of progesterone required to produce this optimum reaction is about 0·75 mg. (0·75 I.U.) of progesterone. It is slightly smaller than the Corner-Allen [1929] unit, and about equal to the Clauberg [1930].

The dosage range over which the response shows proportionality is small. One-half of the above amount produces a questionable reaction, while twice the amount produces a reaction which is nearly maximum. This maximal effect is thus obtained with four times the minimal effective doses. While the data given, depending as they do on visual impressions of size, do not permit of precise calculations of the error of the assay, the restricted dosage range over which the response shows proportionality permits the determination of progesterone with considerable accuracy. The simplicity of the procedures involved, the ready availability of adult rats and the obviation of histologic preparations renders practicable the use of several rats to a dose, thereby increasing the accuracy of assay.

Table I

Daily dose of progesterone mg.	Doses per day	Daily dose of oestradiol μg.	Duration of treatment Days	Decidual response
1·0	2	—	3	+ + + +
1·0	2	0·10	3	+ + + *
0·5	3	—	3	+ + + +
0·5	3	—	3	+ + +
0·5	3	0·05	3	+ + + +
0·5	2	—	3	+ + + +
0·5	2	0·10	3	+ *
0·4	2	—	3	+ +
0·4	2	0·025	3	+ + +
0·25	3	—	3	+ + +
0·25	3	—	3	+ + +
0·25	3	—	3	+ +
0·25	3	—	3	+ +
0·25	3	—	3	+ +
0·25	3	0·03	3	+ +
0·25	3	0·05	3	+ +
0·25	3	0·10	3	— *
0·25	1	0·02	4	+ +
0·25	1	0·02	4	+ +
0·25	1	0·02	4	+
0·25	1	0·02	4	—
0·2	2	0·075	3	+ +
0·13	3	—	3	+
0·13	3	—	3	+
0·13	3	0·03	3	+
0·13	3	0·10	3	— *
0·1	2	—	4	+
0·1	2	—	3	—
0·1	2	0·075	3	+
0·1	2	0·075	3	+
0·1	1	—	4	+
0·1	1	—	4	—
0·1	1	—	4	—
0·1	1	0·02	4	+
0·1	1	0·02	4	+
0·1	1	0·02	4	—
0·1	1	0·02	4	—
0·05	2	—	3	—

* Oestrogen inhibition.

External Uterine Haemorrhage

An interesting incidental finding in these experiments was the frequent occurrence of gross external uterine haemorrhage. In most instances, bright red blood was noted at the vaginal orifice 2 to 5 days after the operation in those animals given progesterone. Of 36 animals in which the phenomenon was sought, gross bleeding was observed in 24. In 14 it was first seen 2 to 3 days after operation, and in 10 it first appeared 4 to 5 days after. There appeared to be a relationship between the degree of decidual

response and the time of appearance of the bleeding. Thus, no bleeding was seen in animals which were not given progesterone; about half of those on the small doses showing a 1 plus reaction bled within 2 days, while half did not bleed. Nearly all animals showing a 2 or 3 plus response bled in 3 to 5 days, while some animals with a 4 plus response had not bled by the 5th day.

The volume of blood lost was difficult to estimate, but it was often comparatively large. If undisturbed, it would sometimes drip from the vaginal orifice on to the cage floor, and usually there was sufficient to stain the adjacent fur. The duration of the bleeding could not be accurately determined from these experiments, for no animals were followed for longer than 5 days. In some, it lasted for but 1 day, while in others it continued for as long as 3 days.

This type of bleeding is very similar to that seen at the end of normal pseudopregnancy in rats which possess deciduomata; bleeding at this time is an indication of decidual breakdown, and heralds the approach of the next oestrous period. Similar bleeding also occurs when the ovaries are removed from deciduoma-bearing rats during pseudopregnancy. The interpretation of this bleeding from progesterone-induced deciduomata may rest upon the partial inadequacy of the injected progesterone in maintaining the growing deciduomata in a normal condition. It is not fully explained by the discontinuity of progesterone action, for it occurred as regularly in animals injected three times daily as in those given single doses daily. The appearance of this haemorrhage in treated rats may be an indication of some progesterone action even when the uterus shows no gross swelling at the time of examination. As a rule, bleeding is accompanied by a decrease in the size of the deciduoma, and after 2 or 3 days of bleeding, small deciduomata may have entirely disappeared.

SUMMARY

The ability of progesterone to promote the development of deciduomata in rats was studied in the following manner. Adult female rats were rendered pseudopregnant by electrical stimulation of the cervix uteri during oestrus. This procedure was successful in 65 consecutive animals. Four days later, the ovaries were removed and the endometrium traumatized by scratching with a needle point. In 22 control animals, whose ovaries remained intact, deciduomata developed in every instance; in 16 other uninjected controls, whose ovaries were removed at the time of trauma, no deciduomata were formed. Progesterone injections in the test animals were begun on the day of operation and continued for 3 days. The degree of decidual response was graded by comparing the gross uterine swelling with deciduoma formed in normal pseudopregnant rats.

A total dose of 1·5 mg. or more gave 3 to 4 plus responses; 0·75 mg. 2 plus; 0·3–0·6 mg. 1 plus or negative. Simultaneously-administered oestrogen in doses totalling 0·3 μg. oestradiol or more inhibited the reaction; smaller doses were without effect. Variation of the volume of oil, or subdivision of the daily dose into two or three injections did not influence the response. The amount of progesterone which, when given over 3 days to animals prepared in this way, will result in a 2 plus decidual response was equivalent to 0·75 mg. (0·75 I.U.).

The author is indebted to Dr. P. G. Weil for permission to include herein some of his data. Professor Hisaw has generously provided the facilities of his laboratories for the major portion of this work; his helpful advice is greatly appreciated.

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OBSERVATIONS ON GROWTH (CHONDRO-TROPHIC) HORMONE AND LOCALIZATION OF ITS POINT OF ATTACK

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ACROMEGALY, dwarfism, and gigantism are generally attributed to pituitary dysfunction. Hypophysectomy is followed by dwarfism, while treatment with alkaline extracts of the pituitary leads to gigantism according to Evans and Long [1921]. Putnam, Benedict, and Teel [1929] found symptoms of acromegaly in dogs after similar treatment, and their findings were confirmed by Evans [1935].

The problem of growth, in spite of these fundamental facts, is far from being solved. Clinical results with growth hormone preparations are disappointing or at least uncertain, and even the experimental gigantism in rats has not been reproduced by others since 1921. Some authorities in this field of research question even the very existence of growth hormone, and Riddle and Bates [1933, 1935, 1938] suggest that certain pituitary extracts owe their action upon growth to 'a balanced combination' of prolactin, thyreo-, or perhaps adrenotropic hormones rather than to a special growth hormone. Reiss [1937] pointed out the importance of corticotropic extracts for growth.

We cannot definitely say whether the growth-promoting effect is due to one substance or not. We can state, however, that our highly purified preparations were practically free of prolactin, cortico-, and thyreotropic activity. An academic discussion may arise from the word 'practically free', when this statement depends merely on the methods and results of comparative assay. It is difficult, however, to believe that prolactin, thyreo-, and gonadotropic potency of a crude extract can be reduced by fractionation 40 to 100 times, without affecting 'growth potency', if the latter should depend on the balanced combination of the discarded fractions.

An assay is reliable when the effect of the hormone can be analysed at a known point of attack. Up to now growth hormone has been assayed by its action upon body-weight of normal rats in a 'plateau' of their weight curve or in hypophysectomized rats. The few suggestions for measuring the size of the body were impracticable on account of technical

difficulties. Growth is commonly assumed to mean an increase in the length of the skeleton. Smith [1930], Koster and Geesink [1929], Dandy and Reichert [1925, 1938], Parkes and Rowlands [1938], and many others drew attention to the lack of skeletal growth after hypophysectomy. Handelsman and Gordon [1930] claim that growth hormone produces periosteal growth of bone. Lucke and Hückel [1933] observed proliferative changes in joint cartilage and regression of the epiphyseal junctions. Mortimer [1937] studied skull growth in rats and in dogs, and in carefully documented papers he states that the vascular supply is interfered with by hypophysectomy, and that the height and breadth of the skull reach normal dimensions, while fronto-occipital growth, especially of the 'face', is impaired. The changes in the teeth, first described by Schour and van Dyke [1932], were not only confirmed, but were also reversed by the administration of purified pituitary extracts. Silberberg [1935, 1936] and Silberberg and Silberberg [1935, 1936, 1937], observed histological changes in the skeleton of guinea-pigs treated with an acid (!) extract of the pituitary. The majority of authors used alkaline solvents when trying to obtain growth hormone. From the limited data available concerning methods of extraction, dosage, and histological features, it may be surmised that Silberberg actually observed the effects of some pituitary substance other than growth hormone. The effects which he observed, hyperplasia and hypertrophy of cartilage, calcification and premature epiphyseal closure, only partially agree with those presented in this paper.

The growth response of hereditarily dwarfed mice and of pigeons to lacto-, thyreo-, and corticotropic extracts and to thyroxin does not disprove the existence of growth hormone. The hypophysis acts upon the trophic condition of somatic and endocrine cells and tissues either immediately or after what may be described as a relay effect through some outlying endocrine gland. Optimal body growth is to a certain extent an 'all or nothing' phenomenon, which surely requires the synergism of all the hormones. That a place in this complex is occupied by growth hormone alone may be shown by localizing and defining a more specific function for the hormone than merely an influence upon the general trophic state of the body. It should then become easier to assess the degree of its separate identity from other hormones which are likewise essential to general trophic normality, but whose specific effect is other than on growth. In this paper we shall attempt to prove that growth hormone is chondrotrophic and that growth effected by means other than chondrotrophic should henceforth be dissociated from the conception of growth hormone.

Normal Tail Growth in Rats

In 851 rats measurements of the tail were made during growth [Levie, 1937]. At birth the tail is 15 mm. long, in adults 180 mm. Growth continues until the rats are 4 months old; the maximal rate of growth is between the 20th and 40th day; during these 20 days the average weight of the rats in our colony rises from 25 to 75 g. The body is about 25% longer than the tail, but as body and tail growth run strictly parallel, the tail length may be assumed to indicate the body growth.

The tail was also studied skiagraphically, using a technique described by Freud and Levie in a previous paper. The total length of the tail and the length and shape of the vertebrae were also observed [Freud and Levie, 1938].

The distance on the skiagram between the first vertebra and the tip of the tail was measured. The first vertebra is crossed by a line which is tangential to the pelvic spinae, and this line served as the medial limit for the measurements.

The tail is composed of 27 vertebrae, of which the last seven are invisible and, according to Donaldson [1924], absent at dissection until the 12th day of life. The early shape of the vertebrae is a quadrangular block, the youngest being very small, and the epiphyses are at first absent. Enchondral ossification begins on the 17th to 20th day. From each vertebral diaphysis two bony nuclei develop proximally, two similar nuclei appearing distally later, and in the first 3 to 5 vertebrae these pairs unite within 27 days to form plates. After the 27th day epiphyseal junction occurs commencing in the 3 proximal vertebrae, and continuing until by the end of the 4th month all epiphysis have developed and closed. From observation of the stage of development of the vertebrae, therefore, the age and size of the rat during the first 4 months of its life may be inferred and the relevant data may conveniently be compiled in the form of a tail calendar. This is of use in assessing the developmental abnormalities which may occur after hypophysectomy or other procedure.

Tail and Hypophysis

In 100 rats, all 45 days old and each weighing 80 g., complete hypophysectomy was carried out in 70 and a dummy operation performed in 30 controls. The modified parapharyngeal technique described by Freud [1938] was adopted. The control operation proceeded to the stage of opening the episphenoidal dura and lesion of the anterior lobe, but this was left *in situ*. Sagittal serial sections of the sella were surveyed to control the operation, and only in a very few were microscopic residues observed.

Immediately after hypophysectomy the tail and vertebral growth

ceased entirely, except for an increase of a few millimetres in those rats which showed a residual pituitary tissue at the end of the experiment. The average tail growth of the control rats was 11 mm. in 7 days and 25 mm. in 21 days, with a deviation of ± 3 mm.

The development of the vertebral epiphyses is disturbed after hypophysectomy. Skiagrams of the tail show a disappearance of the space between epiphyses and diaphyses, the proximal vertebrae preceding the distal in this respect; a young rat assumes within a few days the characteristic appearance of an adult rat. The artificially 'closed' epiphysis in hypophysectomized rats always exceed in number the spontaneously closed epiphyses already present at operation. An illustrative example is shown in our experiment No. 7, in which at operation 12 controls had an average of 6·0 closed epiphyseal junctions, 10 hypophysectomized rats 6·5, whereas the corresponding post-operative values after 7 days were 6·6 and 13·2.

The closure of epiphyses seems final and irreversible by growth hormone. In a series of 6 rats, 15 vertebrae had no growth disks on the 22nd day after hypophysectomy and the remaining 12 vertebrae were in varying stages of transition. After treating these rats for 9 days with a preparation of proved potency, not only was the number of closed vertebrae unchanged, but the number of closed growth disks had increased to 23 and the tail had not grown. Obviously, the irreversible alteration of the epiphyseal growth disks had also involved those vertebrae in which the disk was still visible at the end of the first 22 days. Fig. 1 shows two skiagrams of the tail of (*a*), a hypophysectomized, and (*b*), a control rat, with 14 days' interval between the first and second picture. The tail of the operated rat has not grown and 8 epiphyses are closed; that of the control rat has grown 30 mm., and only 1 epiphysis is closed.

The Action of Growth Hormone

The negative results obtained by delayed treatment after hypophysectomy clearly indicate the necessity of commencing treatment immediately after the operation, if tail growth is to be maintained.

The preparations utilized had been extracted from acetone-dried or fresh beef pituitaries and purified by charcoal adsorption and elution, followed or preceded by isoelectric precipitation, using Dingemanse's method [1938]. Daily doses of 1 to 24 weight increase units were injected intraperitoneally in two equal portions. The assay of weight units was carried out in hypophysectomized rats of 150 g. by daily intraperitoneal injection of the presumptive unit. A preparation of reference was always used and the threshold dose was estimated of this preparation and of the unknown one, this dose producing in the majority of injected rats a 7-g.

gain in 7 days. The comparison forms the basis of calculating the unit strength of the unknown preparation [Dingemanse and Freud, 1935].

The following table shows a few examples of the effect of treatment on the weight and tail-growth of 600 rats. Each rat weighed 80 g., and groups of six received varying amounts of growth hormone over a 7-day period commencing on the day of operation.

Table I

No. of protocol	Prep.	Dose		Increase of tail length	Increase of weight
		Dry res. injected	Aeq. in acetone dry powder		
		μg.	mg.	mm.	g.
38	187	600	1·5	8·2	6·5
42	,,	,,	,,	9·0	2·0
43	,,	,,	,,	8·6	9·0
46	225A	8000	20·0	16·2	18·4
38	225B	32	1·5	7·0	2·7
42	,,	,,	,,	9·0	8·0
51	225C	25	,,	10·3	7·2
51	225D	10	,,	7·3	4·0
Hypophysectomized, untreated			—	0	7·0
Normal	,,		—	11·0	20·0

Table I shows that although the weight increase is very variable, the tail growth varies within narrow limits and is reasonably proportional to the dosage-level. The normal rate of growth is not only attained but even exceeded in the rats under treatment.

The development of the epiphyseal disks remained normal. Daily administration of 12 units kept the epiphyses 'open' even longer than normally. Fig. 2 shows tail growth induced by growth hormone after hypophysectomy, in doses of 1·5 mg. of preparation 225A daily for 14 days. The increase in tail-length was 27 mm. and no epiphyses were closed. Fig. 3 is a comparison of the 10th vertebrae of a normal (*a*), a hypophysectomized untreated (*b*), and a hypophysectomized injected (*c*) rat, treated over a period of 14 days. Vertebral growth is as conspicuous in the normal and the injected rat as arrested growth is in the untreated rat. The skiagram shows the closure (with or without previous growth) of vertebral epiphysis. The growth of the tail-length and the vertebrae are thus valuable indicators of growth hormone activity.

We propose to replace the original technique of assay on a weight basis by a new technique, whereby, in a 7-day experiment commencing on the day of operation, the tail growth is compared with that following the use of standard preparations under the same conditions.

A suitable minimal requirement is 6 mm. average growth in at least 5 rats per group, the deviation permitted being ±1·5. Should the rate of

growth in any rat exceed that of the majority in the group or of others selected at random, it must be examined histologically for residual hypophyseal tissue.

Histology of the Skeletal Growth

A large amount of skeletal material, collected from the above experiments, was subjected to histological analysis. Before discussing the results of the analysis a brief description of the normal longitudinal growth of bones in the rat will be given. During this phase the proliferating cells of the epiphyseal cartilage are arranged in columns and the intercellular matrix is scanty. Towards the diaphyseal primary marrow cavity the cells are distended, a phase preceding the replacement of these cells by vascularized connective tissue, approaching from the marrow and opening the cavities which surround the cartilage cells. In the matrix a network of thin cancellous bone develops and cartilaginous residue is still visible between the developing bony layers. In young rats (7 weeks) the growth disks are already closed on the epiphyseal side by a bony plate consisting of the transverse cancellous bone of the epiphysis. At the end of normal growth the production of cartilage cell columns ceases. The fine structure of diaphyseal spongiosa is destroyed and replaced by massive bone. This is in part transversely arranged, leaning against the epiphyseal cartilage, which is also closed on the diaphyseal side by a bony endplate; the growth disk ceases to exist. The remains of the epiphyseal cartilage has become an epiphyseal disk enclosed by two bony plates. Later the epiphyseal disk gradually disappears and bone is formed in the epiphyseal space. In the majority of the bones of the rat, however, this does not occur. Consequently, closure of the epiphysis refers, in our terminology, to the stage at which the residue of the epiphyseal cartilage (or disk) has become enclosed between transverse bony plates.

In the growth disk or epiphyseal zone of the normal growing rats we find, therefore:

(1) cells arranged in columns, those nearest the marrow cavity being distended;

(2) little cartilaginous matrix;

(3) vascular connective tissue from the primary marrow cavity invading these cell columns;

(4) a fine network of cancellous bone, forming the continuation of the cartilage columns towards the marrow cavity.

Figs. 4, 7, 10, and 13 show the following details clearly:

Fig. 4, the proximal growth disk of the tibia in a rat of 4 weeks.

Fig. 7, a costal bone-cartilage junction in a rat of 7 weeks.

Fig. 10, growth disk of a proximal vertebrae in a rat of 7 weeks.

Fig. 13, schematic drawing of the proximal growth disk of the tibia in a rat of 3 months.

In hypophysectomized rats important differences are to be observed. All the growth disks exhibit the same features; the regular columns of cells, so characteristic of growing cartilage, show little or no development, while distended cells also are arranged differently, forming round groups as if they would prefer transverse proliferation. The mitogenesis of cartilage cells does not seem to have ceased entirely, but the typical arrangement of longitudinal growth is lost. The matrix has increased and is less regularly distributed among the cells. Very characteristic is the straight transverse boundary line between the growth zone and the diaphyseal marrow cavity, the cancellous primary bone appearing to be cut off. The cavities of the cartilage cells are no longer open. One month after hypophysectomy a transverse bony plate closes the epiphysis, from which a number of massive bony processes project towards the diaphyseal marrow cavity. Nothing is left of the fine structure of cancellous bone exhibited by normal rats of a similar age. The picture closely resembles that already described as characteristic of cessation of growth.

It is extremely important to note that, whereas the development of cartilage is gravely impaired by hypophysectomy, the process of bone development remains undisturbed. The structure of bone everywhere is normal, and no instance of a departure from the normal in the progress of bone development was observed. Obviously, therefore, the agents necessary for bone growth are unimpaired by hypophysectomy. This agrees with the already known fact that desmal bone, such as cranial bone, differs in no respect in the hypophysectomized, normal, or injected rat.

To summarize, in the hypophysectomized rat we find the following phenomena:
(1) no 'directed' growth of cartilage;
(2) irregular distribution of cartilage cells;
(3) presence of cell division in cartilage;
(4) increase in the amount of matrix;
(5) closure of the growth zone of the epiphysis by a bony plate;
(6) disappearance of fine primary cancellous bone.

Figs. 5, 8, 11, and 14 illustrate:

Fig. 5, the proximal growth disk of the tibia in a rat of 7 weeks, 14 days after hypophysectomy.

Fig. 8, a costal bone cartilage junction in a rat of 7 weeks, 14 days after hypophysectomy.

Fig. 11, the epiphyseal cartilage of a proximal tail vertebra in a rat of 7 weeks, 14 days after hypophysectomy.

Fig. 14, a schematic diagram of the proximal epiphyseal disk of the tibia in a rat of 3 months, 4 weeks after hypophysectomy.

On histological inspection of bony structures of hypophysectomized rats receiving daily injections of growth hormone, the restoration of normal conditions is evident. The columns of cartilage cells are even more regular and the cells more numerous than in normal controls from the same breed, the growth disks in particular containing more cells. The cartilage cell columns are again extended by primary fine cancellous bone processes in parallel arrangement towards the primary marrow cavity. The sequelae of hypophysectomy are obliterated in all the specimens, as shown by the examples given below.

Figs. 6, 9, 12, and 15 illustrate:

Fig. 6, the proximal growth disk of the tibia in a rat of 7 weeks, 14 days after hypophysectomy and daily treatment with 12 U. of growth hormone (injections commencing on day of operation).

Fig. 9, a costal bone cartilage junction in a rat of 7 weeks, 14 days after hypophysectomy, and daily injections of 12 U. of growth hormone.

Fig. 12, the epiphyseal cartilage of a proximal tail vertebra in a rat of 7 weeks, 14 days after hypophysectomy, and daily injections of 12 U. of growth hormone.

Fig. 15, a schematic diagram of the proximal epiphyseal disk of the tibia in a rat of 3 months, 4 weeks after hypophysectomy, and daily injections of 12 U. of growth hormone.

Summary

1. After hypophysectomy, longitudinal bone growth ceases, especially in the tail, and 7 days after operation the difference between operated and control animals may be readily detected in skiagrams.

2. The epiphyses are closed soon after hypophysectomy.

3. Epiphyseal closure, once completed, cannot be reversed by treatment with growth hormone.

4. Growth hormone treatment, when commenced immediately after hypophysectomy, prevents epiphyseal closure and maintains normal longitudinal growth in the tail.

5. The assay of growth hormone is simple and reliable, using as indicator the tail length and vertebral development as shown by serial skiagrams.

6. A minimal requirement of 6 mm. growth is advised as a basis of comparison between unknown and reference standard preparations.

7. The growth defect after hypophysectomy is definitely localized in the growing epiphyseal cartilage.

8. The histological features of the process of growth cessation after

hypophysectomy are exactly analogous to those exhibited at the end of the normal growth period.

9. Hypophysectomized rats show no alteration in the development of bone tissue, and desmal bone, such as cranial bone, develops normally.

10. Growth hormone has a biologically typical point of attack at the proliferating cartilage and the terms growth hormone and chondrotrophic hormone are therefore synonymous.

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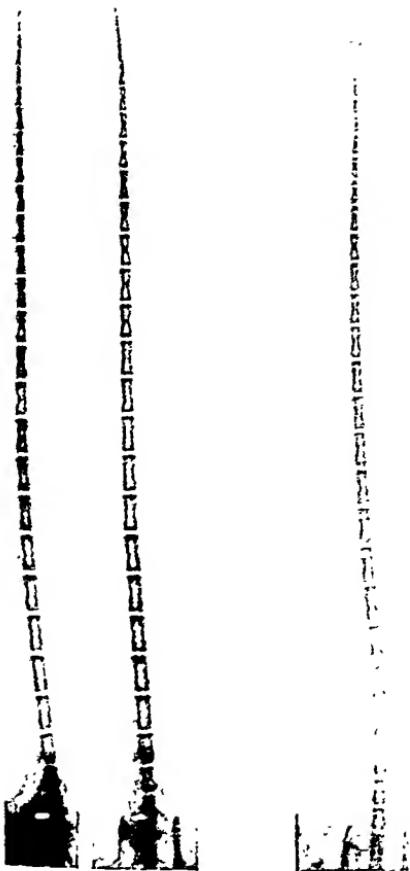
FIG. 1.

FIG. 2.

(a) Skiagrams of the tail of a
hypophysectomized rat with
14 days interval between the
1st and 2nd picture.

(b) Skiagrams of the tail of a normal
rat with 14 days interval between
the 1st and 2nd picture.
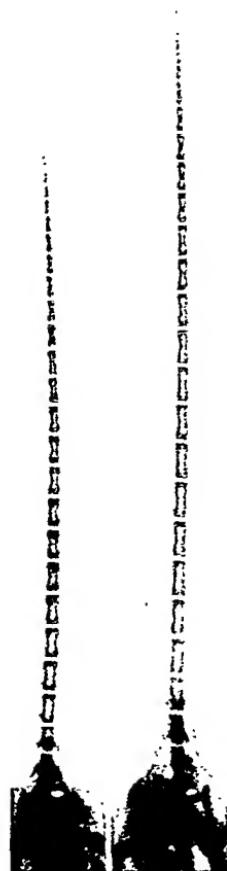
Tail growth of a hypophys-
ectomized rat, treated with
growth hormone during 14
days.
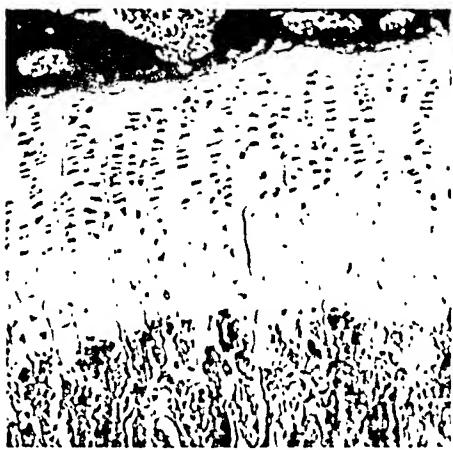
Fig. 4. Tibia, Normal, Mallory. × 94

Fig. 7. Costal bone-cartilage junction, Normal Haem.-Eos. × 55


Fig. 5. Tibia, Hypophysectomized, Mallory. × 94
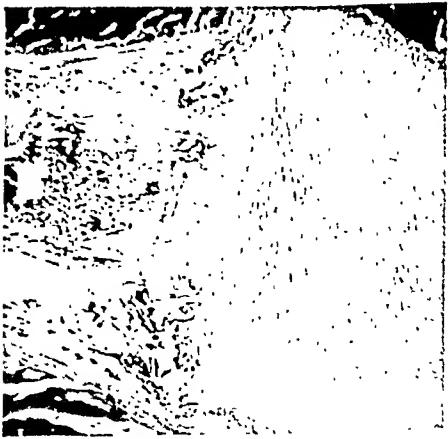
Fig. 8. Costal bone-cartilage junction, Hypophysectomized, Mallory. × 58
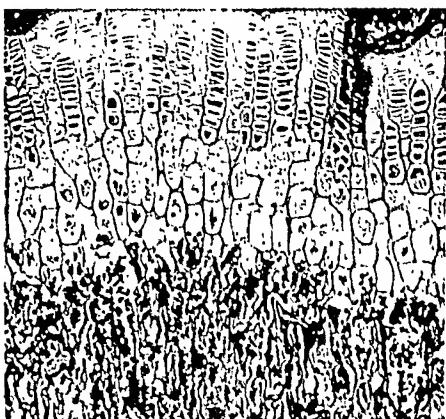
Fig. 6. Tibia, Hypophysectomized treated, Mallory. × 94

Fig. 9. Costal bone-cartilage junction, Hypophysectomized and treated, Haem.-Eos. × 69

Fig. 10. Tail vertebra, normal, Mallory. × 56
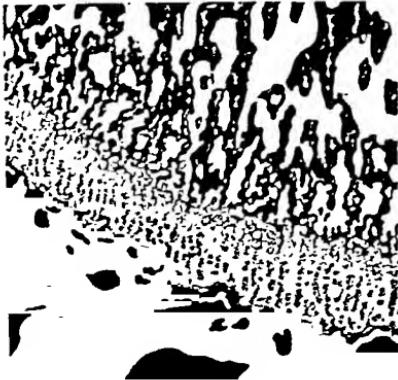
Fig. 13. Schematic drawing of the tibia, Normal, Mallory. × 56

Fig. 11. Tail vertebra, Hypophysectomized, Mallory. × 56

Fig. 14. Schematic drawing of the tibia, Hypophysectomized, Mallory. × 56

Fig. 12. Tail vertebra, Hypophysecto-
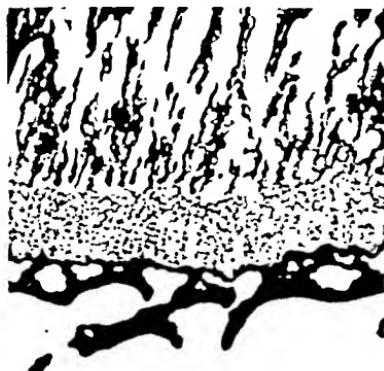
Fig. 15. Schematic drawing of the tibia, Hypophysectomized and treated

PLACENTAL ACTIVITY IN THE MOUSE IN THE ABSENCE OF THE PITUITARY GLAND

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THE function of the placentae in the mouse can be studied by eliminating the other products of gestation on or about the 12th day of pregnancy. It has been shown that in the presence of the retained placentae the remaining week of 'pregnancy' approximates very closely to the normal, in spite of the absence of the growing foetuses. Thus, the placentae are delivered at normal full term, and delivery is accompanied by a loss of weight greatly in excess of that of the placentae and is followed by oestrus. In the interval between foetal destruction and delivery of the placentae the weight added during the first part of pregnancy is maintained and oestrus is suppressed [Newton, 1935; Brooksby and Newton, 1938]. Mammary development and ligamentous transformation of the symphysis pubis [Gardner, 1936] proceed normally, and 19 days after impregnation mammary glands and symphysis pubis are indistinguishable from those at the end of normal pregnancy [Newton and Lits, 1938]. Allowing for some difference in the choice of criteria, the same unimportance of the growing foetuses in maintaining the changes characteristic of the last week of pregnancy is found in the rat [Kirsch, 1938; Klein, 1935; Selye, Collip, and Thomson, 1935; McKeown and Zuckerman, 1938].

With the object of finding whether the activity of the placentae was direct or indirect, Newton and Lits [1938] removed the ovaries shortly after foetal destruction on about the 12th day of pregnancy. They found that in the ensuing week the mammary glands did not undergo involution, and in some cases attained full-term development, provided placentae were retained. If oöphorectomy resulted in placental abortion, as it frequently did, involution of the mammary glands was rapid and extensive. On the other hand, no pubic reabsorption took place in the absence of the ovaries, which appeared also to be necessary for the maintenance of body-weight. These observations agree with those of Selye, Collip, and Thomson [1935], who found full mammary development in the rat after oöphorectomy with placental retention, and are compatible with those of Klein [1935], who found that ovaries as well as placentae were necessary for mucification of the vagina.

The results which follow show the effect—or lack of effect—of hypophysectomy in mice carrying retained placentae, the experiments being complementary to those of Newton and Lits on the effect of oöphorectomy. Mammary development, body-weight, and symphysis pubis were observed.

Methods

Foetal destruction was performed on the 12th day of gestation, without open operation, by lightly anaesthetizing the mothers with ether and bursting the amniotic sacs by digital pressure through the abdominal wall. If complete evacuation of the uterus was desired, the placentae were then dislodged from their attachments, still without open operation, by holding the uterus with the finger and thumb just above each placenta and gently squeezing in a downward direction. In these cases the course of evacuation was not followed in detail, but the uteri were always empty the following day.

Hypophysectomy was performed by the parapharyngeal method of Thomas [1938], which is characterized by a very complete exposure of the pituitary gland. A transverse cut across the basi-occipital bone followed by splitting of the occipito-sphenoidal synchondrosis enables the anterior part of the basi-occipital bone to be removed entire. The pituitary, which is more posteriorly placed than in the rat, is thus completely exposed and the whole of it can be removed by suction under direct vision. The plate of bone is not replaced. Serial sections were made through the appropriate part of the brain and skull in two of the early operations (mice A and B) without any trace of pituitary tissue being found. As the whole gland can be seen to come away cleanly in one piece leaving an empty cavity, and since the latter can be re-explored *post mortem*, no further serial sections were made. The operations were performed under a magnification of 12 or 14 times. The anaesthetic was ether or 0·5 c.c. 5% Nembutal intraperitoneally with ether in addition if necessary.

Sections were made of the same part of the fourth mammary gland of every mouse for histological examination, and the remainder of the glands were dissected from the skin after fixation in Bouin's fluid, stained with haematoxylin, dehydrated, and mounted whole in dammar. For the purpose of indicating the extent of mammary development an image of that part of the 3rd gland near the nipple was projected by appropriate apparatus on to a drawing-board, and its silhouette filled in with indian ink (Fig. 1).

The length of the interpubic ligament was measured with a millimetre scale, the distance between the nearest bony points with the ligament on the stretch being taken. None of the animals had previously been pregnant or pseudo-pregnant.

Weighing was performed at the same time each day, the animal's bladder first being emptied by digital compression.

RESULTS

These are presented in Table I and Figs. 1 and 2. The most important points are as follows:

1. After complete uterine evacuation on the 12th day of pregnancy, or following abortion shortly after this day, profound involution of the mammary glands occurs (Fig. 3 and Fig. 1, M to T). This involution is prevented by the presence of placental tissue in the uterus, as can be seen from Fig. 1, A to L, and Figs. 4 and 5. In most cases further mammary growth takes place (compare Fig. 1, U to W), and though the sections have not been examined carefully for mitoses, these have been noticed from time to time.

2. The day-to-day weights of those animals for which the records are sufficiently complete have been plotted in Fig. 2, the two sets of curves referring to animals with and without placentae respectively. The animals undergoing abortion had lost 20% of their aggregate weight by the 18th day, while those retaining their placentae had lost only 3% by the same date.

3. In all but a single mouse (O) the symphysis pubis failed to open in the group losing placentae. The exception had retained placentae till the 16th day, and its pubes were separated by less than 1 mm. at autopsy on the 19th day. Of the group retaining placentae till the 19th day, only two (B and L) showed a pubic separation of less than 2 mm., and in the case of L this was probably due to the small amount of placental tissue present. Mouse H is regarded as belonging to the 'retained placentae' group.

4. In mice D, G, K, Y, and to a lesser extent X, the alveoli of the mammary glands were full of secretion and frequently distended, and the secretory cells were full of vacuoles. Although a little coagulum was present in the alveoli of all the glands, and vacuolated cells could nearly always be found, and although in some cases the alveoli were moderately full of secretion, the extensive vacuolation in the glands mentioned distinguished them from the others and made them appear like the normal full-term glands of the intact animal. These findings will be dealt with in the discussion, but it is desirable to point out now that if mice D, G, K, X, and Y are omitted altogether from the series, the findings described under headings 1, 2, and 3 still hold good and comprise the evidence which the experiments were designed to obtain. The problems raised with regard to lactation are, however, of some interest in themselves.

Table I

Designation of mouse in Fig. 1	Histological indications of secretory activity of mammary glands	Length of interpubic ligament	Uterus: number of		Day of gestation on which			Symbol of mouse in Fig. 2
			Placentae in	Days empty	Foetuses destroyed	Pituitary removed	Animal killed	
A	A little coagulum in alveoli. Occasional vacuoles in cells	2·0 mm.	8	0	12	13	19	
B	As A	1·0 mm.	4	0	12	12	19	▽•••▽
C	Coagulum in alveoli	2·5 mm.	7–10	0	12	12	18	⊙—⊙
D	Full secretion. Distension of alveoli	3·0 mm.	7	0	12–13	12	19	●—●
E	Coagulum; a few vacuoles; occasional distension	3·0 mm.	7	0	12	12	19	◑—◑
F	Coagulum; occasional distension	4·0 mm.	10	0	12–14	12	19	◪---◪
G	Full secretion. Distension	3·0 mm.	7	0	12	12	19	
H	Coagulum; a few vacuoles	3·0 mm.	(8)	1	12	12	19	
I	A little coagulum	3·0 mm.	5	0	12	12	19	
J	Coagulum	4·5 mm.	4	0	12	12	19	□---□
K	Full secretion. Moderate distension	4·5 mm.	4	0	12	12	19	○•••○
L	Coagulum; a few vacuoles	>1·0 mm.	1–2	0	12	12	19	▼—▼
M	No observation	0	—	5	12	12	19	▽•••▽
N	Some coagulum. Involution	0 (movable)	—	4	12	12	19	▽•••▽
O	Involution	<1·0 mm.	—	3	12	12	19	■—■
P	Involution	0 (movable)	—	6	12	12	19	▼—▼
Q	Involution	0	—	6	12	12	19	○•••○
R	Involution	0 (movable)	—	5	12	12	19	●—●
S	Involution	No record	—	7	12	13	19	
T	Involution	0	—	7	12	13	19	
X	Coagulum; some vacuoles; alveoli uniformly enlarged; not distended	4·5 mm.	3–7	0	12	12	19	×—×
Y	Full secretion; moderate uniform distension	No record	1–5	0	12	12	19	■•••■

Partial records of five further pregnant mice hypophysectomized after foetal destruction on the 12th day of pregnancy and killed on the 19th day have been sent by Miss Beck. In four mice the mammary glands resemble those of mice A, B, and C (Fig. 1), and their average body-weight in grammes on each of the days from 12th to 18th is 26·5, 24, 23·5, 23·5, 23·5, 24, 23. After the post-operative loss the weight is well maintained, and in spite of the absence of any recovery of weight the group of weight-curves resembles the left-hand and not the right-hand set of Fig. 2. In the remaining mouse, 5 out of the 7 placentae were necrotic at autopsy, the mammary glands though not showing involution were less well developed, and an abrupt drop in weight of 3 g. took place on the 15th day. No information is available as to whether the animals had previously been pregnant; this eliminates the interpubic intervals, which were present, from consideration. In one of the mice, mammary alveoli were distended with secretion. The available data substantiate our previous findings.

DISCUSSION

The principal findings require little discussion. The maintenance of body-weight and reabsorption of the symphysis pubis in the presence of retained placentae, phenomena previously shown to be dependent on the simultaneous presence of the ovaries [Newton and Lits, 1938], are found to be independent of the presence of the pituitary. Implicit in these findings is the suggestion that, since the ovaries are still able to play their part, at least part of the synergism between ovaries and placentae is a trophic effect of the latter on the former. This impression is confirmed by the fact that whereas oöphorectomy resulted in abortion in 12 out of 15 mice in the experiments of Newton and Lits, hypophysectomy gave rise to abortion in only six out of 20 mice of the present series. Ovarian function, therefore, is apparently preserved in spite of the absence of the pituitary. Klein, arguing from the state of the endometrium of the rabbit [1933] and of the vaginal epithelium of the rat [1935] during placental retention, is of the opinion that placentae preserve the function of the corpora lutea. Astwood and Greep [1938] have obtained a non-oestrogenic extract of rat placenta which will prolong the life of the corpus luteum. Dr. Ruth Deanesly (private communication) finds that degeneration of the corpora lutea in the ovaries of our animals which aborted their placentae is much more advanced than in those which retained their placentae, though our dates of operation and killing were not the most suitable for conclusive histological observations of this kind.

The question naturally arises whether the claim of Hisaw [Fevold, Hisaw, and Meyer, 1930] to have obtained a substance 'relaxin' from corpora lutea with a specific effect on the symphysis pubis would not repay further investigation. The fact established by Gardner [1936] that large doses of oestrone benzoate over a period of weeks gives rise to ligamentous transformation of the symphysis can hardly be said to explain fully the normal rapid process which occurs in the few days preceding parturition. It is dangerous to assume that it is caused by an entirely different agency, but if it is due to oestrogen it would seem likely that the activity of the latter is catalysed in some way, and the evidence cited in the last paragraph is consistent with an agent of luteal origin. Other problems connected with pubic reabsorption are of great interest, but irrelevant to this discussion. It is, perhaps, important to note its special nature, Gardner and Pfeiffer [1938] having shown that when brought about by injections of oestrogen it is accompanied by actual deposition of new bone in the marrow cavities of the long bones. That it is not entirely independent of the general constitution of the skeleton is suggested by our observation that the batch of mice yielding pubic ligaments 4·5 mm. in length were easy to hypophysectomize on account of the

The five mice in which the secretory activity of the mammary glands was especially noteworthy present an interesting problem. It must be admitted at once that the comparative lack of secretion in such well-developed glands as A, B, and C (Figs. 1 and 5) lends itself readily to the theory that, while growth can be maintained by the placentae, pituitary secretion is necessary for lactation. The appearance of active secretion would then indicate incomplete hypophysectomy. Since we did not make serial sections of the heads of these mice through the pituitary region, we are clearly unable to deny this possibility categorically. In favour of it are the facts that the glands of the oöphorectomized mice of Newton and Lits were secreting, and that mouse H of the present series, which delivered its placentae a day before autopsy, was quiescent. In other words, in the former series presence of the pituitary is associated with mammary secretion, while in the majority of the present series secretion is not marked, and has not been stimulated in mouse H even by the act of delivery.

We have nevertheless included the mice showing active mammary secretion for the following reasons: *First*, any fragments of hypophysis remaining were certainly invisible at a magnification of 14 times, while the pituitary, in full view and appearing about 2 cm. in width, was seen to come out whole. The secretion of the mammary glands was, however, generalized, vigorous, and far from indicative of any deficiency in the mechanism causing it. *Secondly*, Selye, Collip, and Thomson [1933 *a* and *b*] describe in hypophysectomized pregnant rats and mice a transient lactation at the time of parturition capable of keeping alive a litter for about 24 hours. We have confirmed this in mice, our experiments differing only in that the litters of three out of four mice were not completely stillborn. One had a live litter of one, in another one foetus was overlooked during foetal destruction and was born alive; to it were then added two young born of another mother the same day and litter-mates were kept separated from both mother and foster-mother as controls. A third had 8 living young in a litter of 11. All these young had milk in their stomachs after birth, all looked healthy and vigorous, and all were dead in from 24 to 36 hours. The litter-mates of the young taken from a normal mother but kept alone died in less than 12 hours, i.e. about 24 hours before those given to the hypophysectomized mother. *Thirdly*, examination of the whole series of mammary glands shows that secretion, though not marked, is by no means absent in the relatively quiescent members (Table I). All the glands appear capable of secretion, but those under discussion seem to have received some special stimulus. It is tempting to suggest that lactation coincides with parturition and that our routine killing on the 19th day sometimes anticipated natural delivery by a very short time. The findings

in mouse H are not, however, in accord with such a theory, and we can say little more than that the evidence suggests very strongly that a short lactation can occur from some unknown stimulus in the absence of the pituitary. Numerous strains of mice were used in the investigation, and strain differences may have been significant. There are certainly good grounds for a reinvestigation of the factors bearing on lactation.

The point is discussed by Nelson (1936) with the citation of more evidence for and against a transient lactation in hypophysectomized rats, mice, and guinea-pigs, but with much the same conclusion. The criteria of 'lactation' are sometimes open to criticism, and we ourselves have no evidence that the glands of mice D, G, K, X, and Y were capable of yielding actual milk to the exterior. There may be no relation between the histological appearance of the mammary glands A to L, X and Y, and the way in which they would have acted after parturition had the litters been intact and the normal stimulus of suckling applied.

Summary

The presence of placentae in the uterus of the pregnant mouse has been shown to prevent loss of body-weight and to determine reabsorption of the symphysis pubis. Previous investigation has shown these actions to be dependent on the simultaneous presence of the ovaries, but the present work shows that the pituitary is not necessary. Hypophysectomy does not precipitate abortion so readily as does oöphorectomy. A synergic relationship between placentae and ovaries, involving a trophic effect of the former on the latter, is probable.

The presence of placentae in the uterus also has a trophic influence on the mammary glands which is independent of ovaries and pituitary. Certain problems relating to lactation are discussed.

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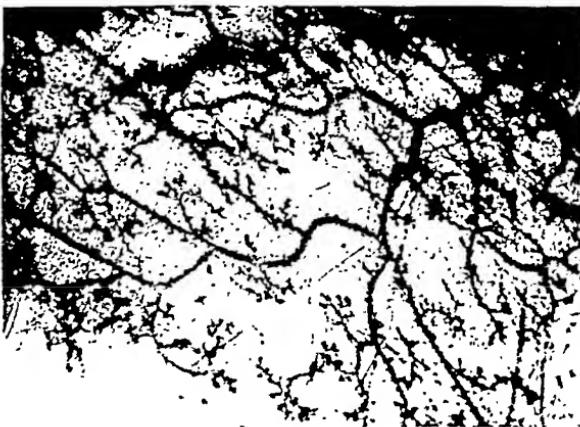
FIG. 3. Photomicrograph of mammary gland of mouse S, 7 days after uterine evacuation, and 6 days after hypophysectomy. × 12½. Whole mount
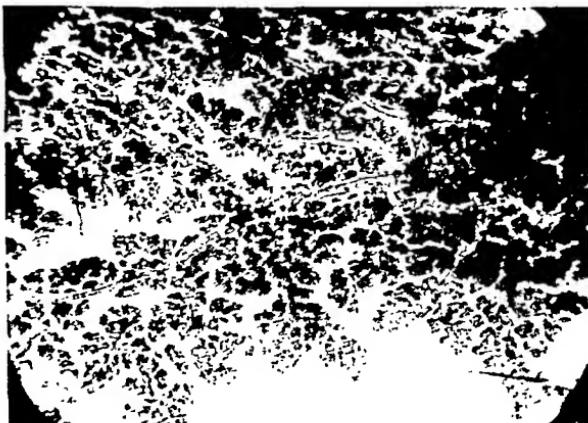
FIG. 4. Photomicrograph of mammary gland of mouse A, having retained placentae, 6 days after hypophysectomy. × 12½. Whole mount
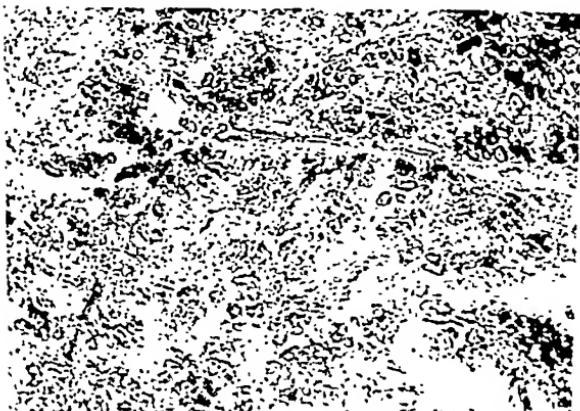
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METHODS OF EXTRACTING COMPOUNDS RELATED TO THE STEROID HORMONES FROM HUMAN URINE

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AT the beginning of biochemical work on male hormones it was assumed that the androgenic material which could be extracted from normal male urine was 'the male hormone', or 'the testicular hormone'. On this supposition, it was hoped that the biological assay of urine extracts would give an index of the activity of androgenic substances in the human body, possibly referable to the level of testicular secretion. It is for this reason that the separation of the maximal androgenic activity as measured by capon comb-growth has been taken as the criterion of a satisfactory extraction in the methods developed by systematic work in various laboratories. It has since been realized, however, that androgenic activity is not specific to one substance, and that at least two related compounds with androgenic activity are present in the mixture of neutral compounds which can be prepared from urine. Further, these two compounds, androsterone and *trans*-dehydroandrosterone (Δ^5-androsten-3(β)-ol-17-one), which can be isolated from the urine of women as well as from the urine of men [Callow and Callow, 1938], have not been found in tissue, whilst the only androgen isolated as yet from testis tissue, testosterone, has not been found in urine. This distinction between hormones and excretory transformation products [cf. the discussion by Callow, 1938] is confirmed by the discovery that testosterone is degraded in the human body to give androsterone and the stereoisomeric, inactive compound, aetiocholan-3(a)-ol-17-one [Callow, Callow, and Emmens, 1939], an observation which is independently confirmed, as regards androsterone, by Cook, Hamilton, and Dorfman [1939]. It must also be considered that 'male' hormone in the urine can be derived from the adrenal cortex, a suggestion first put forward by Simpson, de Fremery, and Macbeth [1936], and supported by the isolation of abnormal quantities of androgens and related steroids from the urine of pathological cases [Callow, 1936a; Burrows, Cook, Roe, and Warren, 1937; Butler and Marrian, 1938; Crooke and Callow, 1939]. While the compounds found in urine are, presumably, present in the circulation for a short time at least, the balance of probability is against their being the original hormones; in

fact, the existence of androgenic activity in human urine extracts is an accident due to the lack of specificity of hormonal activity in relation to chemical structure, and the consequence that some transformation products of the testicular and adrenal cortical hormones possess androgenic activity. In the case of transformation products from the testis secretion, this activity can be assumed to be less, by amounts varying in different compounds, than that of the parent substance; on the other hand, some of the parent substances in the adrenal cortex may not themselves be androgenic. For these reasons the comb growth-promoting activity of urine extracts, which consist of a complex mixture of degradation products, is not by any means a specific index of the production or utilization of androgens in the human body.

A possible alternative method of investigating the excretory transformation products of steroid hormones is provided by a colorimetric method of assay. The colours given by 17-ketosteroids in modifications of Zimmermann's reaction are fairly characteristic and can be used for quantitative determination of this class of compounds. Wu and Chou [1937], who pointed out some of the considerations discussed above, first applied the reaction to a large series of urines. Its application to urine extracts is justified [Callow, Callow, and Emmens, 1938] by the close spectrographic similarity of the colours given by neutral fractions of urine extracts and by pure compounds, and by the fact that 17-ketosteroids, namely, androstan-3(β)-ol-17-one, Δ^5-androsten-3(β)-ol-17-one, aetiocholan-3(α)-ol-17-one, and $\Delta^{3:5}$-androstadien-17-one, have been isolated from urines as probable degradation products of the gonadal or adrenal cortical steroids. The additional circumstance that the comb growth-promoting activity of urine extracts, expressed in terms of the international standard, is roughly proportional to the content of 17-ketosteroid compounds, may, perhaps, be regarded as an indication that, on the whole, the mixture of degradation products varies but little in composition, although its amount may vary. Unfortunately, a chemical test with a greater degree of specificity has not yet been perfected, and, until this problem is solved, there will remain a fundamental difficulty in devising methods for obtaining an optimal extraction of material from urine, when its exact chemical nature is obscure.

In spite of the series of assumptions which must be made in attributing significance to the results of colorimetric assay, the advantages of this method over the biological method are very great with respect to ease of operation, sensitivity, high reproducibility, and low error. For this reason, in the work we have done in examining and comparing methods of extracting urine we have given first place to the colorimetric assay figures. Capon assays have been employed, but are valued more as an indication, where

divergence between the two results is considerable, that factors have been at work to alter the usual compositions of the mixture in the extract.

The inception of the work now described was a sequel to the publication by one of us of a method of 'extraction of male hormone from urine for biological assay' [Callow, 1936b]. A fuller systematic examination of variations in conditions of extraction, with this provisional method as a starting-point, and a comparison with others based on different principles, seemed to be called for in the light of our own experience, and of the claims made for methods published from other laboratories [cf. particularly Gallagher, Koch, and Dorfman, 1935; Peterson, Gallagher, and Koch, 1937; Dingemanse, Borchardt, and Laqueur, 1937; Dingemanse and Laqueur, 1938]. In addition it was hoped to obtain further light on the conditions for extraction of oestrogens from urine. This work was undertaken as part of a scheme of investigation of methods of extraction and assay of substances with hormonal activity from body fluids, under the auspices of the Hormones Committee of the Medical Research Council. The investigation cannot be called exhaustive or even complete, but sufficient has been done to make it clear that the method originally proposed, which differs only in detail from the method of the Chicago workers, is satisfactory, with minor modifications; whilst one other method, which follows closely that of the Amsterdam workers, gives comparable results, and is advantageous when dealing with small quantities of urine to be assayed colorimetrically for clinical diagnostic purposes.

The first method has been applied to the examination of normal levels of urinary excretion by human subjects of 17-ketosteroids and, in a few instances, of androgenic material, and the results are briefly reported.

<div align="center">EXPERIMENTAL</div>

<div align="center">*General Methods*</div>

Conditions of preservation, hydrolysis, and extraction of urines. The urines used in this investigation were mixed collections of men's urine, made either in the National Institute or the Courtauld Institute, or of women's urine, made in the Middlesex Hospital Nurses Home. We wish to express our thanks to Sister Jones for the considerable care and trouble taken in arranging this last collection. The urine, generally preserved with toluene, but occasionally with Brilliant Green, was stored in a cold room until required. The pH was 5·5–6·5. No urine which had been kept more than a day without preservative or which showed any signs of decomposition was employed except, of course, in the experiments dealing with the effects of storage.

The method of extraction which served as a standard for comparison with other methods was the 'provisional routine method' of Callow [1936b],

which was modified in the course of investigation only by providing for a more complete extraction of the phenolic fraction. The modified method with a shorter period of hydrolysis is described in detail at the end of this experimental section. Experiments were carried out to determine the duration of extraction with benzene necessary in the continuous extraction apparatus employed under the usual conditions of operation, and to determine the amount of oestrogenic material removed from the benzene solution by shaking with successive lots of sodium hydroxide solution. No detailed description of these is necessary. The variation in the weight of neutral material and amount of 17-ketosteroid extracted by the routine method was found to be small. A batch of 10 l. of women's urine was divided into four lots of 2·5 l. each. The weights of the neutral fractions of the four extracts were: 98, 92, 104, and 99 mg., and the contents of 17-ketosteroid per litre (see below) were, respectively, 12·1, 13·0, 12·9, and 13·0 mg.

The quality of certain of the materials used was as follows: Concentrated hydrochloric acid, 33% w/w (B.P. 1932); benzene ,'crystallizable'; absolute alcohol, a commercial grade, purified from aldehydes (see below); carbon tetrachloride, 'technical'.

Colorimetric assay of 17-ketosteroids ('sterones'). The method previously described [Callow, Callow, and Emmens, 1938] has been used without modification. The only difficulty which has been encountered is that due to variation in the purity of the absolute alcohol. If, however, commercial 'absolute' alcohol is treated with 4 g. per litre of *m*-phenylenediamine hydrochloride, allowed to stand in the dark for a week, with occasional shaking, and then distilled, rejecting the head and tail fractions [Rowe and Phelps, 1924], the product is a satisfactory medium for the reaction. It has been emphasized before that determinations of the absorption of the reaction-mixture in the green (approximate wave-length 5200 Å) must be accompanied by a measurement in the violet, and that, unless the ratio E_v/E_g is reasonably low (not exceeding 0·8 with Ilford 'Spectrum' filters), it must be assumed that the relative amount of interfering substances present is high in proportion to 17-keto-compounds. Little or no quantitative significance can be attached to figures for the content of '17-ketosteroid' calculated in cases with a high value of E_v/E_g, and they have been enclosed in square brackets in the data which follow.

Biological assay of androgens. The potency of extracts is determined by intramuscular injection of solutions in arachis oil into Brown Leghorn capons, or, rarely, by inunction on to their combs. The oil solutions are made at such a concentration that a suitable quantity of the material being assayed is administered in daily injections of 0·1 ml. Generally, groups of 5 birds are employed. Comb growth is measured by the added

increases in length and height that occur as the result of injections, and is compared with that resulting from the injection of androsterone into a control group of birds at the same time. Details of the methods and the standard curves used are given by Emmens [1939]. The androgenic potency of extracts is expressed as the equivalent in international units of the androsterone standard per litre of urine. The accuracy of a determination varies with the age of the birds used and the amount of comb growth stimulated; the limits of error ($\pm 2\sigma_m$ or $P = 0.95$) are usually, however, within 60–140 %. The effect of augmenting substances is fortunately small in these tests, and more definite significance may perhaps be attached to these biologically determined results than to those dealing with oestrogenic activity.

Biological assays of oestrogens. Determinations of oestrogenic activity have been carried out with ovariectomized mice. A uniform technique has been used, with two injections of 0·1 ml. of oil solution of the extract, one on each of two consecutive days, and the taking of three smears, one on the 3rd day and two on the 4th day after the first injection. Oestrogenic activity is expressed as the equivalent in international units of the oestrone standard per litre of urine. An approximate figure is obtained by injecting a series of different doses of the extract to be assayed, using one mouse per dose. In some cases this estimate, which is correct within about 40–250 %, is sufficiently accurate for our purpose. More accurate estimates are obtained by comparing the responses of groups of 10 or 20 mice simultaneously injected with the extract and with international standard oestrone, at least 20 mice being used for the injections of the standard. The range of error ($\pm 2\lambda_m$ or $P = 0.95$), which is dependent on the number of animals used, is then rarely greater than 70–145 %, and is usually less than this. In the great majority of cases, the figures given here are based on two assays of the material, each with groups of 10 mice. A full description of this method is given by Emmens [1939], together with a discussion of the significance of such assays.

Determinations of oestrogenic potency in terms of a standard are greatly affected both by the particular assay method used and by the presence in the extracts of augmenting substances which are themselves without oestrogenic activity. Some of the differences between the estimated activities of members of the present series of extracts may therefore be due to the presence of varying amounts of augmenting substances, and, in many cases, both the estimates themselves and the ratios between them would almost certainly have been different had another assay technique been employed. The figures recorded here have, therefore, no absolute value, and are comparable only among themselves. Even the validity of this comparison is questionable, for the oestrogenic component is probably

a mixture, and, obviously, extracts of apparently equal oestrogenic potency obtained by different methods might be of very different composition.

Conditions of Hydrolysis of Urine by Acid under Varying Conditions.

Since Adler [1934] demonstrated convincingly that the androgenic substances in urine, like the oestrogenic substances, occur in the form of conjugated, water-soluble compounds, from which they required to be liberated by heating with mineral acid before extraction with fat solvents, this process of hydrolysis forms part of all the recognized methods of extraction. Factors which may be varied are the amount and nature of the acid, and the time and temperature of heating. The effect of these is considered in the following sections.

1. *Amount of acid.* A collection of urine from normal women was divided into a number of equal portions, some of which were brought to pH 1 by the addition of 20 ml. of concentrated hydrochloric acid per litre, whilst 40 ml. of acid per litre were added to the others. They were then boiled under reflux for periods varying from $\frac{1}{4}$ hour to 16 hours, and extracted with benzene in a continuous extraction apparatus. The experiment with 1 hour's boiling with 40 ml. of acid per litre was carried out in duplicate, and in addition, one lot was extracted without boiling. The neutral fraction from the benzene extract was examined colorimetrically. The values for mg. of 17-ketosteroid per litre, given in Table I, show that for all periods of boiling the greater yield is obtained with the higher concentration of acid.

Table I. *Effect of concentration of acid on liberation of 17-ketosteroids from normal women's urine on boiling*

Time of boiling (hr.)	Content of 17-ketosteroids (mg./l.)	
	20 ml./l. HCl	40 ml./l. HCl
0	—	[1]
0·5	2·6	5·7
1	3·4	6·3, 6·5
2	[2·4]	6·2
4	3·4	5·6
16	3·6	4·2

As preliminary experiments had indicated that a further small increase in the amount of acid does not increase the yield of androgens, and as the deleterious effect of a great excess of hydrochloric acid was to be feared [cf. Butenandt, Dannenbaum, Hanisch, and Kudszus, 1935; Dingemanse and Laqueur, 1938], the amount of hydrochloric acid was standardized at 40 ml. per litre while the effect of varying other factors was investigated. Preliminary experiments showed that the substitution of sulphuric acid for hydrochloric acid is not advantageous, and no further trials were made.

2. *Time of boiling.* The experiment described above indicated that the yield of 17-ketosteroids reached a maximum with 1 hour's boiling and then slowly decreased. This effect required confirmation, particularly in view of the work of Peterson, Gallagher, and Koch [1937], who found that the best time of hydrolysis was 15 minutes for normal male urine treated with 100 ml. hydrochloric acid per litre. For this reason the effect of time of boiling was investigated with several batches of urine from normal men and women. Biological assays of androgens with capons and of oestrogens with mice were carried out in addition to the colorimetric assays. The values obtained for urine of men are given in Table II, and those for urine from women in Table III. These are presented graphically in Figs. 1 and 2 respectively, with the exception of the oestrogen values, which are shown together in Fig. 3.

Table II. *Effect of time of boiling with 40 ml. of HCl per litre on liberation of 17-ketosteroids, androgens, and oestrogens from the urine of normal men*

	Time (hr.)	17-ketosteroids mg./l.	Androgens I.U./l.	Oestrogens I.U./l.
Batch I	0	[2]	ca. 4	<4
	0·25	8·5	62	7
	0·5	8·2	79	13
	1	9·9	74	27
	2	8·8	51	21
	4	8·3	40	16
	8	7·4	40	27
	16	9·9	45	27
Batch II	0	[3·1]	<1	<5
	0·25	10·1	23	20
	0·5	12·5	50	22
	1	11·2	36	24
	2	8·6	28	33
	16	8·5	25	21

The neutral fractions from the two series of hydrolyses of men's urine give concordant results, showing maxima of comb growth-promoting activity at 0·5 hour, with a corresponding maximum in the colorimetric value in 1 case; the divergence in the other case is probably to be ascribed to experimental error. On the other hand, the three series of hydrolyses of women's urine show some irregularity, but the general tendency is for the colorimetric value, after having risen rapidly after 0·25 hour's boiling, to continue to increase slowly. The androgen values are rather discordant. It is evident that the results may vary from one batch of urine to another owing to the operation of some unrecognized factor, but there does seem to be a significant difference in the type of curve given by urines from the two sexes in these experiments. A possible explanation would be that men's urine differs from women's urine in that it contains a ketonic
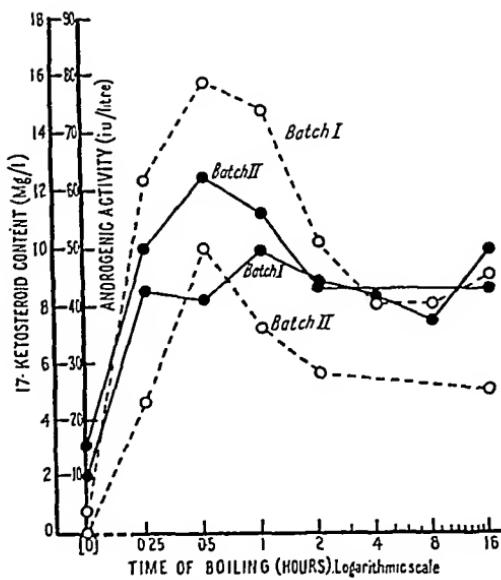
FIG. 1. Liberation of 17-ketosteroids (——●——) and androgens (---o---) from men's urine after boiling for different periods with hydrochloric acid (40 ml./l.).
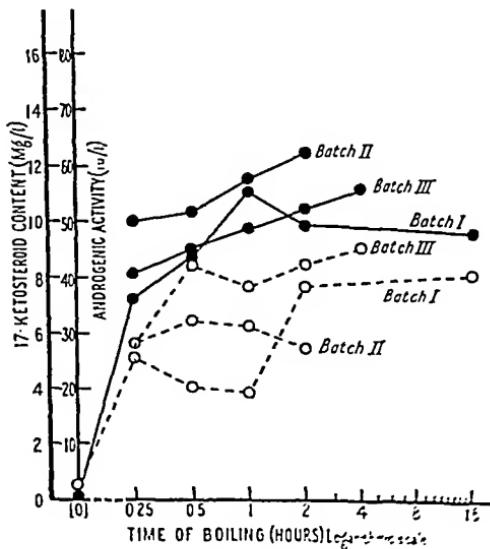

FIG. 2. Liberation of 17-ketosteroids (——●——) and androgens (---o---) from women's urine after boiling for different periods with hydrochloric acid (40 ml./l.).

androgen which is unstable when boiled with acid. Under the conditions of collection the women's urine consisted chiefly of morning specimens, whilst the men's urine was passed during working hours, but this circumstance does not suggest any grounds for the difference. Other factors which might play a part are the greater liability of collections of women's urine to microbial infection and possible minor differences in technique,

Table III. *Effect of time of boiling with 40 ml. of HCl per litre on liberation of 17-ketosteroids, androgens, and oestrogens from the urine of normal women*

	Time (hr.)	17-ketosteroids mg./l.	Androgens I.U./l.	Oestrogens I.U./l.
Batch I	0	[0·3]	ca. 2·5	22
	0·25	7·2	25·5	97
	0·5	8·7	20	108
	1	11·0	19	121
	2	9·8	38	145
	16	9·5	40	160
Batch II	0·25	10·0	28	52
	0·5	10·3	32	78
	1	11·5	31	65
	2	12·4	27	85
Batch III	0·25	8·1	28	—
	0·5	9·0	42	—
	1	9·7	38	—
	2	10·4	42	—
	4	11·1	45	—

since the women's urine was worked up in one laboratory and the men's urine in the other. Peterson, Gallagher, and Koch [1937] record considerable variation in the level, but not in the type, of the time of hydrolysis curve for men's urine, but their only observation on normal women's urine was a comparison of a 0·25-hour hydrolysis of the urine of one woman with 2-hour hydrolyses of the urine of three others, which, when the likelihood of individual variation is taken into account, gives no useful information.

The rather limited data for the oestrogenic activity of the phenolic fractions indicate an increase in the amount of oestrogenic material liberated with increased time of boiling, with a maximum probably occurring after 16 hours. There is a clear difference between the values of the oestrogenic activity in the material liberated from women's and from men's urine, the best yields from the former being of the order of 160 I.U. per litre and from the latter about 30–40 I.U. per litre. This proportion is roughly the same with various times of hydrolysis of the two kinds of urine. Considering that the yield of androgens from these bulk collections is somewhat lower for women than for men, this is in general agreement with the results of Gallagher, Peterson, Dorfman,

enyon, and Koch [1937], who find a marked and consistent difference
1 the ratio of androgens to oestrogens excreted by the two sexes. Their
An/Es' ratio, where 'An' designates international androgenic units per
ay, and 'Es' designates oestrogenic activity as μg. of oestrone per day,
; of the order of 7 for men and 2 for women. The differences in the two
atios are even more markedly in the same direction in our own results,
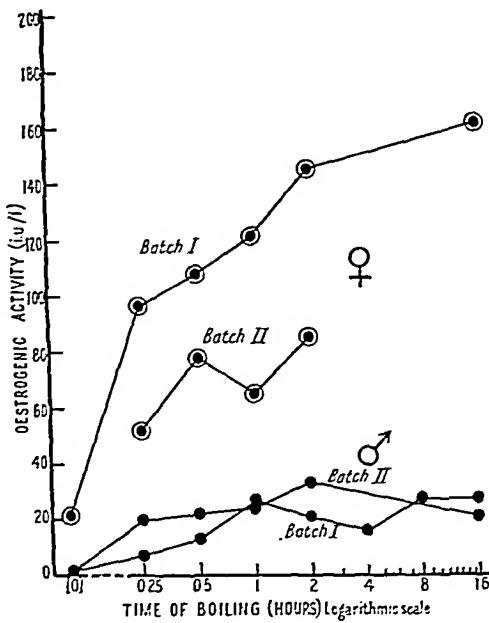
Fig. 3. Liberation of oestrogens from urine of
men (—●—) and women (—◉—) after boiling for
different periods with hydrochloric acid (40 ml./l.).

but in view of the variation from one collection to another, and the
uncertain significance of the figures for oestrogen content, it does not
seem profitable to calculate the ratios, with the implication that they
represent standard figures.

3. *Effects of higher temperature.* In view of the more efficient liberation
of oestrogens from pregnancy urine when acid hydrolysis was carried out
in an autoclave at a pressure of 15 lb./sq. in. (121° C.) observed by Cohen
and Marrian [1934, 1935], experiments were carried out with both men's
and women's urine. Portions of urine from the same batches used for the
boiling experiments (Batch II, Table II, and Batch I, Table III) were
treated with 40 ml. of hydrochloric acid per litre and heated in an auto-
clave at 20 lb./sq. in. (126° C.) for periods varying from 0·5 to 4 hours. The
results are shown in Table IV.

Table IV. *Effect of time of autoclaving at 126° C. with 40 ml. of HCl per litre on liberation of 17-ketosteroids, androgens, and oestrogens from the urine of normal women (Batch Ia) or normal men (Batch IIa)*

	Time (hr.)	17-ketosteroids mg./l.	Androgens I.U./l.	Oestrogens I.U./l.
Batch Ia	0·5	9·6	46	129
	1	8·3	28	169
	1·75	9·2	47	146
	4	9·5	19	136
Batch IIa	0·5	9·7	21	34
	1	11·3	30	34
	2	9·1	20	41
	4	9·1	30	29

An additional experiment with men's urine autoclaved at 15–18 lb./sq. in. for 0·25 hour gave a product with 3·3 mg./1. of 17-ketosteroids as compared with a control, boiled for 1 hour, which gave 8·7 mg./1. Comparison of the figures in the tables shows that the maximum yields of 17-ketosteroids from autoclaved urine are below those from boiled urine. The figures for androgens are irregular, and no significant difference is obvious. As regards oestrogens, it seems that autoclaving is advantageous, as it is with the urine of pregnant women, a period of 1 or 2 hours giving maximum yields. Probably this is the best general method for liberating oestrogens from either men's or women's urine.

The Effects of Putrefaction.

Marrian [1933], and Cohen and Marrian [1934] observed that allowing urine of pregnancy to putrefy was a more effective method of liberating oestrogens than hydrolysis by acid for 1 hour at 100°C., and Patterson [1937] based a routine method of chemical diagnosis of pregnancy on liberation of oestriol by incubation of urine with *Bact. coli*. For this and other reasons connected with the possible effects of putrefaction on steroids in urine, a series of preliminary experiments was made.

The yield of oestrogenic material from normal men's urine extracted by Patterson's method, or by a modification in which the inoculum included *Micrococcus ureae*, was, respectively, rather lower than, or about the same as, that obtained by the routine acid hydrolysis. This may be taken as an indication that the oestrogen of male urine includes material resembling oestriol as well as oestrone [cf. Dingemanse, Laqueur, and Mühlbock, 1938]. The matter was not, however, investigated further.

Several experiments were carried out on the effect of allowing urine to putrefy at room temperature before acidifying to pH 1, adding a further 20 ml. of hydrochloric acid per litre, boiling, and extracting by the routine method. The yield of oestrogens obtained in this way was equal to

the best obtained by any other method. Thus, a specimen of normal women's urine, kept for 7 days without preservative, yielded an extract with 161 I.U. of oestrogenic activity per litre, as compared with 169 I.U. per litre obtained from fresh urine of the same batch by autoclaving for 1 hour, or 160 I.U. per litre by boiling for 16 hours. Analogous results were obtained with specimens of men's urine. On the other hand, the effect of putrefactive decomposition on the values found for 17-ketosteroids by colorimetric assay, and for androgens by capon assay in the material extractable after acid hydrolysis, to which our attention was chiefly directed, was irregular but generally adverse. A preliminary investigation of the chemical changes taking place during putrefaction showed that rotted urines, in contrast to fresh urine, always contained comparatively large amounts of 17-ketosteroids and androgens in the material which could be extracted directly by ether or benzene without acid hydrolysis. The subsequent further yield of 17-ketosteroids and androgens obtained by acid hydrolysis of the extracted urine was low. This effect was concordant with the assumption that the ketones are liberated from their water-soluble combinations by bacterial action, as is the case with oestriol. Whether further chemical change occurs is not known, but it would seem very likely, in view of the work on oxidation or reduction of C_{19} steroids by micro-organisms [see, for example, Schramm and Mamoli, 1938; Mamoli and Vercellone, 1938; Ercoli, 1939]. The data obtained in this series of preliminary experiments are set out in Table V.

One test was made of the effect of preliminary extraction of normal men's urine by ether or benzene on the subsequent yield of 17-ketosteroids and androgens after hydrolysis with acid. The figures obtained, given at the end of Table V, indicate that no 17-ketosteroid is removed by extraction before hydrolysis. The androgen content is not significantly lowered by ether extraction, and the apparent rise after benzene extraction is most readily explained by error in the biological assay.

Although previous mild putrefaction does not greatly affect the colorimetric assay of the neutral portion of an extract of acid-hydrolysed urine which has had no preliminary extraction, gross differences outside the limits of normal variation of different samples sometimes occur. It is, therefore, a matter of practical importance to know how urine samples intended for diagnostic assay should be preserved. Also, in the course of the work being carried out at the National Institute for Medical Research on the isolation and recognition of steroids from particular types of urine, it has been considered essential that no decomposition should have taken place, so that there may be greater certainty that the compounds obtained are actually those originally excreted in the conjugated form. A reaction of the urine more alkaline than pH 7 is the first indication of possible

Table V. *Effects of putrefaction on the extractable 17-ketosteroids, androgens, and oestrogens of normal urine*

Material and treatment	17-ketosteroids mg./l.	Androgens I.U./l.	Oestrogens I.U./l.
Women's urine. Stored 7 days at room temperature. Boiled 1 hr.+acid.	10·3	19	161
Women's urine; same batch. Fresh. Boiled 1 hr.+acid. (Cf. Table III.)	11·0	19	121
Men's urine. Stored 13 days at room temperature. Boiled 1 hr.+acid.	8·9	12	30
Men's urine; same batch. Fresh. Boiled 1 hr.+acid.	11·6	31	26
Men's urine. Stored 3 months at 0° C., then 3 months at room temperature. Boiled 1 hr.+acid.	—	25	11
Men's urine; same batch. Fresh. Boiled 1 hr.+acid.	—	49	9
Men's urine. Stored 7 days at room temperature. Boiled 1 hr.+acid.	9·1	23	35
Men's urine; same batch. Fresh. Boiled 1 hr.+acid.	11·2	36	22
Men's urine. Stored 10 days at room temperature. Evaporated to ⅓ bulk. Extracted with ether. Ether extract.	7·7	8·5 (ketonic fraction)	—
Remainder boiled 1 hr.+acid. Benzene extract.	[1·2]	<1	—
Men's urine; same batch. Fresh. Evaporated to ⅓ bulk. Extracted with ether. Ether extract.	[0·4]	<2	—
Remainder boiled 1 hr.+acid.	6·2	23	—
Men's urine. Fresh. Evaporated to ⅓ bulk. Extracted with ether. Ether extract.	1·4	1·4	—
Remainder boiled 1 hr.+acid.	10·3	27	—
Men's urine. Stored 14 days at room temperature. Extracted with ether. Ether extract.	[4·4]	6	—
Remainder boiled 1 hr.+acid.	[4·3]	11	—
Women's urine. Stored at room temperature until putrid. Extracted with ether. Ether extract.	4·7	22	—
Remainder boiled 1 hr.+acid.	[3·8]	15	—
Women's urine. Kept at 37° C. for 96 hrs. Extracted with ether. Ether extract.	[6·0]	11	—
Remainder boiled 1 hr.+acid.	[4·9]	12	—
Women's urine; same batch. Fresh. Boiled 1 hr.+acid.	8·6	17	—
Men's urine. Brought to pH 1 and extracted with ether. Ether extract.	0	0·1	—
Remainder boiled 1 hr.+acid.	12·8	34	—
Men's urine; same batch. Brought to pH 1 and extracted with benzene. Benzene extract.	[0·9]	0·4	—
Remainder boiled 1 hr.+acid.	11·0	63	—
Men's urine; same batch. Hydrolysed with acid directly.	11·8	44	—

decomposition. In the light of the data given above, the existence of free 17-ketosteroids extractable without hydrolysis also leads to suspicion that decomposition has occurred. Contrary to Broster, Allen, Vines, Patterson, Greenwood, Marrian, and Butler [1938] and Butler and Marrian [1938], we have not found appreciable quantities of free 17-ketosteroids or androgens in any fresh urine except in cases where the content of combined steroids was very large indeed.

The process of putrefaction is uncontrollable and uncertain, and, unfortunately, the preliminary experiments we have carried out with different preservatives gave somewhat equivocal results. It is clear that a preservative must be added in order to stop bacterial action, but that in any event the sample must be worked up as fresh as possible. Phenolic preservatives, e.g. 'tricresol' or thymol are a nuisance in the fractionation of the extract. Brilliant Green (1% of a 10% aqueous solution) has been used, but interferes with colorimetric determination of the pH, and gives rise to highly-coloured extracts. Toluene (1%) is generally used, and seems satisfactory, provided that it does not come into contact with rubber or 'composition' stoppers. Neither of the last two preservatives stops putrefaction for long, and there is reason to suspect that enzymic decomposition occurs in their presence without any change in the acidity of the urine being apparent. Dingemanse [1932] observed unexplained increases in the content of extractable oestrogen in men's urine after storage, even when sterile. We have made similar observations.

Other Methods of Extraction.

Brief mention may be made of trials with certain methods of extraction of urine proposed primarily for the extraction of oestrogens. The first is that of Smith and Smith [1935], which is a variant of the general acid-hydrolysis and benzene extraction method. Normal women's urine was boiled for 15 minutes with 150 ml. of hydrochloric acid per litre and then extracted. As the oestrogen assay described by Smith and Smith [1935] is carried out on an unfractionated extract, the opportunity was taken to examine the content of 17-ketosteroids, androgens, and oestrogens in the whole extract, and of the separated phenolic and neutral fractions. The values obtained are shown in Table VI, compared with values for urine from the same batch extracted by the routine method.

The figures demonstrate the existence in the total extract before fractionation of much ketonic material which produces general absorption in the colour reaction for 17-ketosteroids, and also of material augmenting the oestrogenic activity of the phenolic fraction [Emmens, 1939], more in the Smith-Smith extract than in the control. As the yield of 17-ketosteroids was slightly higher and that of androgens and oestrogens apparently

significantly higher in the neutral and phenolic fractions respectively of the batch extracted by the routine method, no further investigation was done.

Table VI. *Comparison of the 17-ketosteroids, androgens, and oestrogens in different fractions obtained with much acid and short hydrolysis, or with little acid and long hydrolysis*

Treatment	Fraction	17-ketosteroids mg./l.	Androgens I.U./l.	Oestrogens I.U./l.
150 ml. HCl/1. Boiled 15 min.	Total	[23·3]	18	150
	Neutral	10·6	29	<1·5
	Phenolic	—	—	75
Control: 40 ml. HCl/1. Boiled 1 hr.	Total	[21·6]	49	110
	Neutral	11·8	46	<1·5
	Phenolic	[1·3]	—	96
50 ml. H_2SO_4/1. Boiled 15 min. Bu$_2$O extraction.		—	10·8	30	52
Control: 40 ml. HCl/1. Boiled 1 hr.		—	14·5	49	44

In the course of this work we were informed, through the courtesy of Dr. Erwin Schwenk, of work being done by Drs. D. R. McCullagh and T. R. McLin on the use of dibutyl ether as an extracting agent for androgens and oestrogens. Their early method consisted of a combined hydrolysis and extraction in which the urine, brought to pH 1 with sulphuric acid, was stirred at 50° C. for periods of 30 minutes with three successive portions of dibutyl ether. In our hands this process gave a yield of material which was negligible compared with a routine extraction (cf. Table VII). The method published by McCullagh and McLin [1938] consisted of hydrolysis of the urine by boiling for 15 minutes after adding 50 ml. of concentrated sulphuric acid per litre, and extracting by agitation with two successive lots of 200 ml. of dibutyl ether per litre. This method was much more promising (cf. Table VI), but not better than the control except in respect of extraction of oestrogens. A preliminary experiment showed that the dibutyl ether available commercially required to be purified before use, since it contained impurities which gave a strong general absorption with the colour-reagent for 17-ketosteroids.

The method of hydrolysing urine with acid in the presence of a fat solvent, with the idea of removing the liberated material from the destructive influence of the acid, seems to have been first used by Siebke [1930] and Funk and Harrow [1930]. Kemp and Pedersen-Bjergaard [1933] employed this method, and more recently Borchardt, Dingemanse, and Laqueur [1934] published an improved version of the method in which an adequate amount of acid was used; the method was applied to the extraction of androgens [Dingemanse, Borchardt, and Laqueur, 1937], and the superiority of their technique was supported by later studies [Dingemanse and Laqueur, 1938].

Dr. K. Pedersen-Bjergaard kindly informed us, during this work, of the details of his current method, in which carbon tetrachloride was used as the extracting solvent instead of benzene. The use of carbon tetrachloride has the practical advantage that the mixture can be boiled on a water-bath, and the lower-boiling liquid forms the bottom layer, and there

Table VII. *Liberation of 17-ketosteroids, androgens, and oestrogens from urine by simultaneous acid hydrolysis and extraction.*

Material and treatment	17-ketosteroids mg./l.	Androgens I.U./l.	Oestrogens I.U./l.
Women's urine. pH 1. Extracted at 50° C. with 3×85 ml. Bu$_2$O/l.	[1·7]	Trace	*ca.* 24
Control: Women's urine; same batch. 40 ml. HCl./l. Boiled 1 hr., then extracted (benzene).	11·4	29	57
Men's urine. Acid to Congo Red. Boiled+benzene; 3×2 hrs.	[3]	9	*ca.* 6
Men's urine; same batch, pH 1. Boiled+CCl$_4$; 3×2 hrs.	3·9	13	*ca.* 6
Control: Men's urine; same batch. 40 ml. HCl/l. Boiled 1 hr., then extracted (benzene).	11·0	23	19
Women's urine. 20 ml. HCl/l. Boiled+CCl$_4$; 4×2 hrs.	6·5	16	37
The same. 40 ml. HCl/l.	8·1	17	43
„ „ 100 ml. „	11·6	23	62
„ „ 140 ml. „	11·4	27	73
Control: Women's urine; same batch. 40 ml. HCl/l. Boiled 1 hr., then extracted (benzene).	11·0	32	86
Men's urine. 120 ml. HCl/l. Boiled+CCl$_4$; 4×2 hrs.			
1st extract.	7·7	35	*ca.* 16
2nd „	2·7	4	—
3rd „	[1]	0·6	—
4th „	[0·6]	*ca.* 0·15	—
Total.	11·0	40	
The same. Combined extracts.	11·8	35	15
Control: Men's urine; same batch. 40 ml. HCl/l. then extracted (benzene).	11·3	23	27

is no danger of bumping or of fire. Dr. Dingemanse and her co-workers, as an alternative to benzene, used trichloroethylene, but carbon tetrachloride has the advantage of being a saturated, stable substance available in fairly pure form. We have studied the influence of the concentration of acid, and also of the number of times successive lots of extracting liquid are used, on the yield of 17-ketosteroids, androgens, and oestrogens. Our results are entirely concordant with those of Dingemanse *et al.* [1937].

The first trials, made with men's urine, showed that when the urine was made acid to Congo Red with hydrochloric acid, and boiled under reflux with three successive lots of benzene for periods of 2 hours, poor yields were obtained. Slightly better results were obtained with carbon tetrachloride

and acidification to pH 1, but the yields were still well below those from the routine method of acid hydrolysis and subsequent benzene extraction, as is obvious from the data in Table VII. In another experiment,
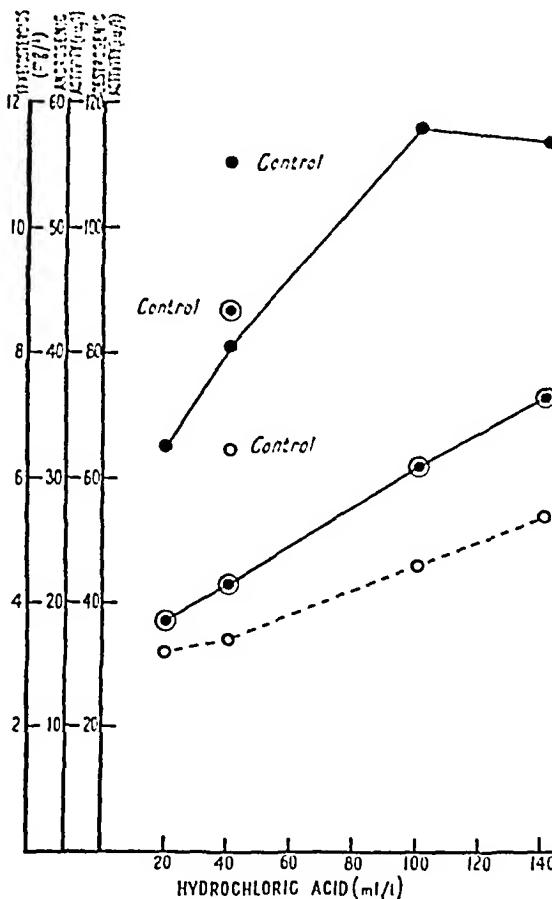
Fig. 4. Liberation of 17-ketosteroids (—●—), androgens (---○---), and oestrogens (—◉—) from women's urine in presence of carbon tetra-chloride and varying amounts of concentrated hydrochloric acid.

a batch of normal women's urine was divided into five lots: one of these was hydrolysed and extracted by the routine method, the other four were treated with hydrochloric acid in the proportions of 20 ml. per litre (giving pH = 1) and 40, 100, and 140 ml. per litre, and boiled for 2-hour periods with four successive lots of carbon tetrachloride. The values obtained are shown in Table VII and in the diagram (Fig. 4), and demonstrate clearly the advantage of using a large proportion of acid. The yields from the control extraction and the carbon tetrachloride extraction with the highest proportion of acid do not differ significantly. In a further experiment, using men's urine, with 120 ml. of hydrochloric acid per litre, comparison

was made with a control routine hydrolysis and extraction, and the quantities of 17-ketosteroid, androgen, and oestrogen in the four successive carbon tetrachloride extracts were measured. The values obtained are in Table VII. As some 99% of the 17-ketosteroids and androgens are contained in the first three extracts, a fourth extraction is unnecessary. It is seen that the carbon tetrachloride process actually has an apparent advantage over the routine process in respect of 17-ketosteroid and androgen extraction, though not in oestrogen extraction. This has been confirmed as regards 17-ketosteroids by further experience in comparing the two methods with pathological urines.

Proposed Standard Routine Methods of Extraction of Urine

As the result of this exploration of the effect of variations in the method of hydrolysis and extraction, it was concluded that a satisfactory extraction of 17-ketosteroids and androgenic material could be carried out either by a modification of our own provisional routine method or by a modification of the method of Dingemanse *et al.* In the former case the only important change is the reduction in the time of hydrolysis to $\frac{1}{2}$ hour instead of 1 hour, and in the latter the use of carbon tetrachloride as solvent. It is hoped that the following detailed account of these methods will be useful. Our view that the problem of estimation of urinary oestrogens is in an unsatisfactory state has already been discussed, and it follows that no optimal method of extraction can yet be laid down. The extraction of oestrogens by the following methods is admittedly incomplete, but it is probable that useful comparative data can be obtained, provided that only large differences are considered to be significant.

Method I. Separate hydrolysis and extraction. (Modification of the 'routine' method [Callow, 1936b].)

Hydrolysis and extraction. The proportion of concentrated hydrochloric acid (33% w/w) required to bring the urine to pH 1 is first determined with a 100 ml. portion; with a normal, fresh urine at pH 6–6·5, about 20 ml. per litre is necessary. Methyl violet is used as indicator, the colour produced by the urine mixed with an equal volume of the indicator solution being compared with that produced by decinormal hydrochloric acid (the 'Capillator' technique is convenient).

The urine is placed in a flask fitted with a ground-in reflux condenser and a tube from the top of the condenser leading to an arrangement for absorbing fumes in alkali, and is then brought to the boil before the acid is added, and, if toluene is present, the steam is sucked off by a side-tube below the condenser until no more oily drops distil over. Concentrated hydrochloric acid is then added through the condenser in amount sufficient to bring the urine to pH 1, plus 20 ml. per litre in excess. After boiling

for $\frac{1}{2}$ hour, the urine is then cooled, transferred to a continuous extraction apparatus and extracted with benzene ('crystallizable') for 12–24 hours, according to the efficiency of the extractor The benzene extract is then treated as described in the second part of Method II (below).

Method II. Combined hydrolysis and extraction. (Modification of the method of Dingemanse, Borchardt, and Laqueur [1937]).

Hydrolysis and extraction. The urine is placed in a flask fitted with a ground-in reflux condenser, and concentrated hydrochloric acid 33% w/w) is added in the proportion of 150 ml. per litre, and carbon tetrachloride in the proportion of 125 ml. per litre. The mixture is boiled on the water-bath for 2 hours, and then, as soon as it has cooled sufficiently, the lower layer of carbon tetrachloride is sucked out through a tube leading to a collecting flask attached to a filter-pump. The carbon tetrachloride is transferred to a separating funnel and any urine washed back into the flask. The extraction is repeated in the same way, for 2-hour periods, twice more. The carbon tetrachloride is distilled off from the united extracts, and the residue is taken up in benzene in the proportion of 100 ml. per litre of original urine.

Fractionation. The benzene solution is freed from acid by washing twice with 25 ml. portions of saturated sodium bicarbonate solution, freed from phenols by extracting 5 times with 20 ml. portions of 2N sodium hydroxide, and washed with water. These quantities of sodium bicarbonate and hydroxide solutions are suitable for 1 litre of urine. For larger quantities the amounts are increased proportionately. Should phenolic preservatives have been used, the extraction with sodium hydroxide must be repeated after acidification of a portion of the extract produces no cloudiness. The benzene solution is evaporated to dryness and the residue is extracted 4 or 5 times with 5–10 ml. portions of redistilled ether. The ethereal extracts are filtered through a Jena No. 2 sintered-glass filter, and the filtrate is evaporated on the water-bath in a small, weighed flask in a stream of nitrogen until no more readily volatile material remains. The residue is taken up in aldehyde-free, absolute alcohol, generally at a concentration of the equivalent of 0·1 litre of urine per ml. or 0·1 day's output of urine per ml. This solution is suitable for colorimetric assay in the case of most urines. The amount required for biological assay may be measured out and evaporated to dryness, sufficient arachis oil is added from a syringe to the residue to make the volume up to the desired total, and the mixture is heated and stirred until all the oil-soluble material has dissolved.

The phenolic fraction is worked up as follows for assay of oestrogens: First the sodium bicarbonate extract (acid fraction) is back-extracted twice with 20 ml. lots of ether and the ether used for extraction of the phenolic

fraction (below). The combined sodium hydroxide extracts (main phenolic fraction) and water washings are acidified to litmus by the addition of concentrated hydrochloric acid, and then extracted with 3 lots of 30–50 ml. of ether. The ether is washed twice with 20 ml. lots of water, dried over sodium sulphate, and evaporated to dryness in a small, weighed flask. The residue is taken up in alcohol at a convenient concentration, say the equivalent of 0·1 litre per ml. or 0·1 day's output per ml. For biological assay appropriate quantities of this solution are measured out and evaporated to dryness in a stream of nitrogen, and sufficient arachis oil is added to the residue from a syringe to make up to the required volume, and the mixture is heated and stirred until all the oil-soluble material has dissolved. If phenolic preservatives have been used in the urine, it is necessary to steam-distil the final residue and then extract it with ether; the extract is then washed, dried, and evaporated in the usual way.

Extraction of 17-ketosteroids and Androgens from the Urine of Individual Patients.

In view of the experience with bulk collections, it is clear that when urines of individual patients are to be examined, either for purposes of diagnostic assay or for the isolation of characteristic constituents, conditions of preservation must be carefully attended to, and the urine must be hydrolysed as soon as possible. Further, although it will be a commonplace to those dealing with urine for other purposes, the necessity of obtaining complete 24-hour specimens and of calculating results on a 24-hour basis must be emphasized. We have dealt with urines from patients with outputs ranging from 0·3 to 4·5 l. per day. Moreover, in common with other constituents, the 17-ketosteroids are present in higher concentration in the morning urine than in that passed during the rest of the day. An investigation of one woman patient showed that over a period of 6 days the average total excretion per day of 17-ketosteroid was 5·7 mg. and of this 2·5 mg. was excreted in 420 ml. of morning urine and 3·2 mg. was excreted in 1,100 ml. of urine during the rest of the day.

In agreement with other workers [Gallagher, Peterson, Dorfman, Kenyon, and Koch, 1937] we have found that there is considerable variation in 17-ketosteroid and androgen excretion between one individual and another, and that the values for one individual may vary considerably from one period to another. With the object of determining standards of normality, we examined the 17-ketosteroid excretion, and in some cases the androgen excretion, of a series of men and women with no obvious sexual dysfunction in the Courtauld Ward of the Middlesex Hospital. We have to thank Dr. J. D. Robertson for arranging these collections. The figures obtained are given in Table VIII.

The variation of values of 17-ketosteroid excretion is very large in both sexes, the range for separate samples from men being 3·5–15·0, whilst for those from women it is 1·7–12·6 mg./day. From an inspection of the figures in Table VIII, it is clear that the samples from one individual give, on the whole, values which are closer together than values for random

Table VIII. *17-ketosteroid and androgen excretion of patients with normal sexual function*

Sex, age, and disease	Length of periods of each collection Days	17-ketosteroid excretion (mg./day) and androgen excretion, in parentheses (I.U./day) in successive periods	Average 17-ketosteroid excretion ng./day
M., 40, thyrotoxicosis	6	10·4 (59), 9·7, 12·0	10·7
M., 24, psoriasis	6	12·3 (53), 12·0, 13·8	13·1
	5	14·3	
M., 21, haematemesis	6	13·0 (58), 12·5, 14·3, 15·0, 12·9	13·5
M., 46, psoriasis	6	6·7, 6·0	6·4
M., 39, psoriasis	6	9·3, 7·7	8·5
M., 38, thyrotoxicosis	6	6·4 (>44)	6·4
M., 50, haematemesis	6	6·6 (28), 7·0, 8·8	7·5
	5	8·4	8·4
M., 38, melaena	6	3·5 (6·5), 8·3, 10·8, 12·3 (17), 9·8, 8·6	8·9
M., 37, melaena	6	8·2, 8·8, 7·9	7·7
M., 36, haematemesis	6	6·2, 6·9, 10·0	
	3	9·0, 9·1, 7·2, 10·6	8·4
F., 28, psoriasis	6	7·2 (46), 7·9	7·6
F., 34, psoriasis	6	11·1 (34), 9·2, 8·4	9·9
F., 21, obesity	6	11·4 (31), 8·3	9·9
F., 21, psoriasis	6	10·0, 9·8, 9·9	9·9
F., 24, obesity	7	4·0 (25)	4·0
F., 32, thyrotoxicosis	6	5·0, 2·4 (ca. 2), 1·7	3·0
F., 35, psoriasis	7	6·9 (ca. 7), 6·9	6·9
F., 20, thyrotoxicosis	7	6·9, 4·5, 2·9	4·8
F., 38, thyrotoxicosis	7	3·0	3·0
F., 67, achalasia of cardia	3	5·0, 3·6, 3·2, 5·0, 4·0	4·2
F., 23, duodenal ulcer	3	12·6 (20), 9·4	11·0

samples from different individuals. In calculating mean values for men and for women, it is therefore best to determine a mean for each group from the average figures for each individual, thus avoiding the giving of undue weight to the readings from any one individual. The mean figure for men is 9·05 mg. per day of 17-ketosteroid, with $\sigma = 2·416$ for the average figures; that for women is 6·75 mg. per day of 17-ketosteroid, with $\sigma = 3·268$. Fisher's '*t*' test shows that the difference between these means cannot be regarded as statistically significant, as t is equal to 1·921, giving $P > 0·05$, for 20 degrees of freedom.

SUMMARY

The possible origin and significance of the presence in urine of androgenic material and of 17-ketosteroid compounds related to the androgens is

discussed. Although, as yet, products derived from the gonadal or adrenal cortical secretions cannot be distinguished by specific tests, a case can be made out for using biological assay or, preferably, colorimetric assay as an index of the amount of these products as a whole.

As a result of a systematic survey of the effect of varying, within certain limits, conditions of hydrolysis of human urine, two methods of extraction have been adopted which appear to give optimal yields of the 17-keto-steroid compounds. The first method, that of successive hydrolysis and extraction, is suitable for large-scale work, whilst the second method, that of hydrolysis in presence of an extracting solvent, following the Amsterdam method, is convenient for diagnostic assays.

Biological assay of oestrogenic activity is an unsatisfactory method of examining the mixture, consisting of oestrogens and material augmenting their activity, obtainable from urine. The conditions for extraction of oestrogens have, therefore, only been considered incidentally. It has, however, been found that, even with admittedly incomplete extraction, extracts from normal women's urine have much greater oestrogenic activity per litre of original urine than those from men's urine.

Conditions applying to the collection of urine from human subjects are considered, and some preliminary results are given of the examination of individual variation in levels of 17-ketosteroid excretion by adults of both sexes. In the subjects examined, the limits of variation for the two sexes overlapped, and, although men gave higher average values than women, the difference was not statistically significant.

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THE EFFECT OF THE ADMINISTRATION OF TESTOSTERONE PROPIONATE ON THE URINARY EXCRETION OF COMPOUNDS ALLIED TO THE STEROID HORMONES

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INTRODUCTION

THE present investigation originated in a scheme of work on the therapeutic value of androgens in prostatic hypertrophy, undertaken under the auspices of the Therapeutic Trials Committee of the Medical Research Council. In co-operation with Dr. A. W. Greenwood of the Institute of Animal Genetics, and Dr. J. M. Robson of the Pharmacological Laboratory, University of Edinburgh, the androgen and oestrogen excretion of elderly men was investigated before, during, and after treatment with androgens, and a summary account of this work was given by one of us in a lecture [R. K. Callow, 1938]. Technical difficulties allowed the preparation of satisfactory extracts from only a few patients, and in these the changes in androgen or oestrogen excretion with moderate doses of androgen (up to 40 mg. of testosterone propionate per week by injection) were not statistically significant. Bühler [1933] and Kochakian [1937] reported small increases in the androgenic activity of the urine after administration to men of urine concentrates or of androstenedione, but the differences were small and their results are, in addition, subject to the criticism that the methods of extraction they employed were inefficient; thus Kochakian estimates the normal 'male hormone' excretion of young men at about one-quarter of the value now accepted by ourselves or other workers in the field [cf. Dingemanse, Borchardt, and Laqueur, 1937; Gallagher, Peterson, Dorfman, Kenyon, and Koch, 1937]. McCullagh, McCullagh, and Hicken [1933] administered a urine concentrate to a eunuch and reported the presence of androgenic activity in extracts of his urine in the days immediately following, whereas none had been detectable before.

The preliminary conclusion from the investigations in this laboratory was that there is almost complete destruction of administered androgens in the human body, as measured by the index of androgenic activity of urine extracts. Subsequent investigations, now described in detail, which were carried out with the generous co-operation of several clinicians, showed

that, with a sufficiently high level of administration of testosterone propionate, the urinary excretion of steroid compounds underwent marked changes which could be recognized in extracts, either by biological assay of the comb growth-promoting activity or by colorimetric assay [Callow, Callow, and Emmens, 1938]. In the meantime, Dorfman and Hamilton [1939] have reported a study of the urinary excretion of androgenic material after intramuscular and oral administration of testosterone propionate to 2 men showing symptoms of deficient testicular secretion. They made the interesting observation that oral administration of high doses led to a high level of excretion of androgenic activity in the urine, but with poor clinical effect in comparison with $\frac{1}{8}$th to $\frac{1}{4}$th the dose administered intramuscularly, which was accompanied by a comparatively lower androgenic activity of the urine extracts. These results were followed by an investigation of the nature of the active compound in the urine, and the isolation of androsterone was reported [Cook, Hamilton, and Dorfman, 1939] at the same time as our own preliminary communication [Callow, Callow, and Emmens, 1939]. Our own observations, given in detail in the experimental part of this paper, are confirmatory of and supplementary to those of Professor Cook and the American workers. We have studied a series of 6 male patients, of whom 2 were cases of mild prostatic enlargement, 1 was a case of acromegaly, and 3 were eunuchs. Extracts of the urine have been examined during administration of testosterone propionate by intramuscular injection, or, in one case, by implantation of a tablet of the solid material under the skin, and during control periods. The comb growth-promoting activity, the content of 17-ketosteroids and the oestrogenic activity of these extracts have been measured, and, finally, it was possible in one case (the acromegalic, Case 3 below) to isolate androsterone and aetiocholan-3(α)-ol-17-one in such large amount that there is little doubt that these compounds are direct degradation products of testosterone. The details of this isolation are described elsewhere [N. H. Callow, 1939].

<div align="center">SUBJECTS AND EXPERIMENTAL METHODS</div>

Case Histories.

Case 1. A man, aged 58, showing symptoms attributed to prostatic enlargement. The patient, under the care of Dr. Raymond Greene, was given a series of injections of testosterone propionate, 200 mg. per week for the first 3 weeks, 100 mg. per week for the next 5 weeks. Urine was collected for periods of 1 week before treatment began, during the treatment at a level of 100 mg. per week, and after conclusion of treatment.

Case 2. A man, aged 71, showing symptoms attributed to prostatic enlargement. The patient, under the care of Dr. Raymond Greene, was given a series of injections of testosterone propionate amounting to 50 mg. per week. Urine

was collected for periods of 1 week, before treatment began, and in the 4th week of treatment.

Case 3. A man, aged 29, suffering from acromegaly. A detailed case report is given by Schrire and Sharpey-Schafer [1938] under the heading 'Case 3. H.A.W.' No symptoms of adrenal dysfunction were observed. In the course of investigations of the effects of hormone treatment on the urinary creatine and creatinine output, the patient was injected with 10 mg. of oestradiol benzoate daily for 10 days. After a further 18 days, a trial injection of testosterone propionate was given, and after 4 days 100 mg. of testosterone propionate were injected daily for 8 days. Urine was collected during the last 7 days of this treatment, and again, 10 days after the cessation of treatment, for a period of 4 days.

Case 4. A man, aged 45, completely castrated some 10 years ago on account of tuberculosis. He was completely impotent, with atrophied penis. The patient was under the care of Dr. S. Levy Simpson. A complete collection of urine was made over a period of 6 days. Three weeks later, when the patient was receiving 50 mg. of testosterone propionate per day by intramuscular injection, urine was collected over a 4-day period, and, a month later, over a period of 4 days, on the first and last of which 50 mg. of testosterone propionate had been injected.

Case 5. A man, aged 19, who had had one testis removed when he was 13, and the other when he was 14, i.e. just before or during the early days of puberty. He had an infantile penis and secondary sexual characteristics were absent. The patient was admitted to St. Bartholomew's Hospital under Dr. A. W. Spence. A complete collection of urine was made over a period of 3 days. Then a collection was made over a period of 6 days, on the 1st and 4th of which the patient had received injections of 50 mg. of testosterone propionate. Injections were discontinued and a tablet of testosterone propionate was implanted in the patient. Urine was collected for a 6-day period, beginning 9 days after the end of the previous collection.

Case 6. A post-puberal eunuch, aged 38, castrated as the result of a war wound at the age of 19, and now showing negligible evidence of sexual function. A full record of the case has been published by Dr. G. L. Foss [1937, 1938, 1939], who was treating the patient. After a series of injections of testosterone propionate amounting to a total of 940 mg. over a period of 3 months, and after an interval of 11 days, the patient received injections of 20 mg. and 50 mg. on 2 successive days. A complete collection of the urine was made in the 3 days immediately following this, and again, over two 3-day periods 2 months later, after the patient had had a rest from treatment for about 5 weeks. A further collection was made over a 5-day period 2 months later. The patient had received injections of 50 mg. of testosterone propionate and 0·05 mg. of oestradiol benzoate on the day before collection began and received injections on the 2nd and 4th days of 20 mg. and 40 mg. of testosterone propionate and 0·05 mg. of oestradiol benzoate.

Collection and preservation of urine. All urine was collected over complete periods of 24 hours; it was immediately transferred to storage vessels containing 1% of their volume of toluene or of a 10% aqueous solution of Brilliant Green and kept in a cool place until it could be sent to the

laboratory, where it was worked up without delay. All of the samples for which data are given had pH 6–6·5 and showed no evidence of putrefactive changes.

Extraction. Urines were hydrolyzed by boiling for 1 hour after the addition of 40 ml. of HCl per litre. The product was extracted with benzene and the extract, after removal of acids, divided into phenolic and neutral fractions, of which the former was assayed for oestrogenic activity and the latter for 17-ketosteroids and androgenic activity. The method of extraction and fractionation was that described by Callow [1936] for urines from cases 1 and 2, and the modification of this method described by Callow, Callow, Emmens, and Stroud [1939] for the remainder. One sample each from cases 4 and 6 was extracted with benzene before hydrolysis in order to examine the free steroid compounds, and then the extracted urine was hydrolyzed and again extracted to yield the steroid compounds originally present in water-soluble, combined form.

Colorimetric assay of 17-ketosteroids. This was carried out by the modified Zimmermann reaction as described by Callow, Callow, and Emmens [1938]. The colour produced in this reaction with 17-ketones of the steroid group of compounds (the 17-ketosteroids) has a characteristic absorption band in the green, and, subject to the condition that no large amounts of interfering substances are present, the absorption of light in this region of the spectrum, measured on a Hilger 'Spekker Photoelectric Absorptiometer' with an Ilford 'Spectrum Green' filter, is taken as a measure of the 17-ketosteroids present in the neutral fraction of urine extracts. The values obtained are expressed in terms of mg. of 17-ketosteroids per day's output of urine, calculated by reference to a calibration curve obtained by measurements with pure androsterone, and indicate the amount present of a mixture of steroid compounds which may include androsterone, aetiocholan-3(a)-ol-17-one, Δ^5-androsten-3(β)-ol-17-one (*trans*-dehydroandrosterone), and other similar compounds.

Biological assay. Androgens are determined by intramuscular injection of arachis oil solutions of the neutral fractions of the extracts into capons, or, rarely, by inunction on to their combs. Groups of 5 birds are employed, and the increase in comb size (length+height) is compared with that occurring in a control group receiving androsterone at the same time: the final estimate is determined by reference to standard curves. The values obtained are expressed in terms of international units of androgenic activity per day's output of urine.

The oestrogenic activity of the phenolic fractions of urine extracts are determined by the injection of arachis oil solutions into ovariectomized mice and examination of vaginal smears. A preliminary estimate is made by the injection of graded doses into a series of single mice, and the final

determination on a group of 10 or 20 mice, comparison being made with a group receiving pure oestrone. The values obtained are expressed in terms of international units of oestrogenic activity (oestrone standard) per day's output of urine. For reasons which have been discussed elsewhere [Callow, Callow, Emmens, and Stroud, 1939], these figures have only a comparative significance, since the method of extraction used is not the most efficient possible, and, moreover, the evaluation of the activity of crude extracts in terms of oestrone depends upon the exact technique of assay.

A full description of the methods of assay and a consideration of the errors involved has been published by one of us [Emmens, 1939].

RESULTS

The values determined for the daily excretion of 17-ketosteroids, androgens, and oestrogens by the different subjects, when under treatment and during control periods, are set out in Table I, which is self-explanatory.

DISCUSSION

The rise in androgenic activity of extracts of the urines of these patients during intramuscular administration of testosterone propionate is in general agreement with the results of Dorfman and Hamilton [1939]. The rise in oestrogen excretion confirms observations by Steinach, Kun, and Peczenik [1936] on rats, and by Steinach and Kun [1937] on men, when androgens are administered. The nature of this excess oestrogen is indicated by the circumstance that it passes into the phenolic fraction of the urine extracts, but there are no other clues to its composition, and it seems unlikely that it is present in sufficient quantity to be readily isolated. There is no evidence whether this oestrogenic material is a direct degradation product of testosterone, or is secreted by some endocrine organ as a result of stimulation by the testosterone. Since the effect is observed in eunuchs, the testis is not responsible, but the adrenal cortex may be. Laroche, Simonnet, and Bompard [1939] did not observe any increase in the urinary excretion of oestrogen by ovariectomized women receiving testosterone propionate, but their paper lacks detail, and it is difficult to judge how sensitive their methods were, and whether the gonadectomized female organism is, in fact, different from the gonadectomized male organism in this respect. The failure of Kochakian [1938] to find any oestrogens in the urine of dogs receiving androgens by injection suggests a species difference in steroid metabolism.

In cases 1 and 2 the androgen excretion during testosterone administration rose to approximately the level for normal men from about $\frac{1}{3}$ of this figure. The oestrogen excretion showed an approximately ten-fold

Table I

Case no. and description	Treatment	Period of collection Days	Urine examination		
			17-ketosteroids: Colorimetric assay mg./day	Androgens: Capon assay I.U./day	Oestrogens: I.U./day
1. Prostatic enlargement	None	7	—	15	ca. 4
	100 mg. T.P. per week	7	—	54	44
	After treatment had ceased	7	—	30	22
2. Prostatic enlargement	None	7	—	17	<3
	50 mg. T.P. per week (4th week)	7	—	46	ca. 24
3. Acromegaly	100 mg. T.P. per day	1	31·5	110	130
	„	1	33·8	—	—
	„	1	41·8	—	—
	„	1	42·6	105	1360
	„	1	28·0	—	—
	„	1	40·6	—	—
	„	1	45·7	163	274
	None: 10–13 days later	1	9·1	12	25
	„	1	10·6	ca. 8	—
	„	1	11·7	—	29
	„	1	7·2	—	—
4. Eunuch	None	6	3·1	10	<1·5
	50 mg. T.P. per day	4	11·6	81	38
	50 mg. T.P. 1st and 4th days	4	[2] (free) 10·8 (combined)	0·4 (free) 34 (combined)	1 (free) 36 (combined)
5. Eunuch	None	3	10·9	18	12·5
	50 mg. T.P. 1st and 4th days	6	13·4	50	16
	Implanted tablet of T.P.	6	9·8	25	16
6. Eunuch	20 mg. and 50 mg. T.P. on 2 previous days	3	[2] (free) 30·7 (combined)	<2 (free) 176 (combined)	<4 (free) 65 (combined)
	None	3	8·4	21	8
	„	3·5	7·2	15	6
	Beginning the day previous to first collection, on alternate days, 50, 20, 40, and 40 mg. T.P. and 0·05 mg. oestradiol benzoate	5	15	45	75

T.P. = Testosterone propionate.
[ ] = Nominal figures of atypical colour reactions.

increase. In case 3, during testosterone administration, the excretion of 17-ketosteroids was about 4 times, and the androgenic activity of the extracts roughly 12 times, that during the subsequent control period. The values for oestrogen excretion, as far as they have been determined, show increases of from 5 to 50 times. The figure for the 4th day of collection is surprisingly high. Even if the figure on the 1st day of collection were assumed to be affected by the course of treatment with oestradiol benzoate ending 24 days before, which is not very probable, testosterone propionate administration is still able to increase it considerably. The fact that the patient was suffering from acromegaly, and that this malady is often accompanied by disturbance of adrenal cortical function, must be considered in this case. The low ratio of androgenic activity to 17-ketosteroid content of the urine extracts may possibly be connected with this, but it is, to say the least, unnecessary to assume that in this case alone the large changes observed were the result of a mechanism involving an abnormal function of the adrenal cortex for which there is little or no evidence. The three eunuchs, cases 4, 5, and 6, were receiving lower doses, and the rises in the urinary products, with intramuscular injection, although less spectacular, are quite definite.

In case 6, receiving at one period 0·05 mg. of oestradiol benzoate on alternate days, it appears that only a small proportion of the activity of administered oestradiol is recoverable in the urine. This is in accordance with the results of other investigators [Mazer and Israel, 1937; Kemp and Pedersen-Bjergaard, 1937], though no work can be said to have been done on human subjects with thoroughly efficient methods of extraction.

The suggestion that examination of the excretory transformation products might give some indication of the efficiency with which administered androgens were utilized cannot be sustained after the report by Dorfman and Hamilton [1939] of the low therapeutic effect but high androgen excretion following oral administration of testosterone propionate. It is of interest, in this connexion, to compare the low levels of androgen and 17-ketosteroid excretion in case 4, when administration of testosterone was by an implanted tablet, with the higher levels observed with intramuscular injection, and it is quite in accordance with expectation that efficient utilization should occur when the hormone is supplied at a constant moderate level to the organism, whilst an intermittently excessive supply should lead to rapid destruction and excretion of the transformation products, without more marked hormonal effects [Deanesly and Parkes, 1937].

As very high levels of androgen excretion without administration of hormones have been found only in cases of hyperplasia or tumour of the adrenal cortex, as women normally excrete nearly as much androgenic material as men, and as castrates of both sexes have been found to excrete

amounts of androgenic material which may approach normal levels, there was some ground for the supposition that the adrenal cortex might be responsible for most, or even for the whole, of the androgens in the urine. The primary object of the present investigation was to obtain some clue to the part of the urinary excretion of androgens or of 17-ketosteroids which could be regarded as derived from the testis. The result has been to show that testosterone, the compound regarded as the characteristic secretion of the testis, does in fact yield androgens and 17-ketosteroids as excretory transformation products. The chemical examination of the extracts from the urine of case 3 [N. H. Callow, 1939] shows that they contain an excess, compared with extracts from normal men's urine, of androsterone and its stereoisomeride aetiocholan-3(α)-ol-17-one, but that *trans*-dehydroandrosterone, a urinary androgen occurring in large quantities in urine from adrenal tumour cases [R. K. Callow, 1936 b; Crooke and Callow, 1939] is not present in recognizably increased amount. The conclusion is that one course of the degradation of testosterone in the human organism can be represented by the scheme:

| Testosterone | Androsterone | Aetiocholan-3(α)-ol-17-one |
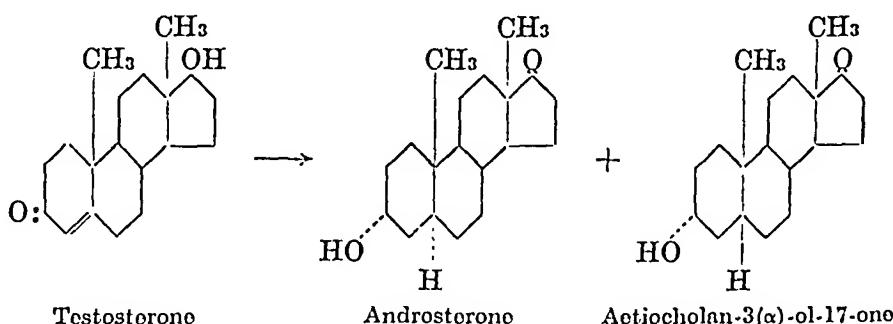
The reduction of the 3-keto-group to a 3(α)-hydroxyl group and of the 4:5-ethylenic linkage to give both of the possible configurations at position 5 is exactly similar to the process of reduction undergone by progesterone [Hartmann and Locher, 1935]. The oxidation of the 17-hydroxyl group may be the result of an enzymic mechanism specific to this group.

Unfortunately, neither androsterone nor aetiocholan-3(α)-ol-17-one can yet be regarded as degradation products characteristic of endogenous testosterone. Androsterone is found in the urine of normal women [Callow and Callow, 1938], and aetiocholan-3(α)-ol-17-one has been isolated from the urine of a case of adrenal hyperplasia in a woman [Butler and Marrian, 1938]. In either case it would be bold to speculate on the possibility that testosterone is the parent substance.

SUMMARY

The administration of testosterone propionate to male or castrated male human subjects by injection in amounts of 50 mg. per week and upwards

results in an increased urinary excretion of 17-ketosteroids, and of androgenic and oestrogenic material.

Our most grateful thanks are due to the clinicians, Dr. G. L. Foss, Dr. Raymond Greene, Dr. E. P. Sharpey-Schafer, Dr. S. Levy Simpson, and Dr. A. W. Spence, who placed the urine from their patients at our disposal and took so much trouble in arranging for its collection, and have allowed us to quote from the case histories.

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THE INFLUENCE OF THE SUBCUTANEOUS IMPLANTATION OF TABLETS OF SOLID INSULIN ON THE BLOOD SUGAR LEVEL OF THE RABBIT

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THE prolongation of the action of subcutaneously administered insulin has obvious advantages in clinical practice, and the introduction of long-acting 'protamin-insulin' [Hagedorn, Jensen, Krarup, and Wodstrup, 1936] and 'zinc protamine-insulin' [Scott and Fisher, 1937] has been of great value in the treatment of *diabetes mellitus*.

Experiments with the sex hormones have shown that when many of these substances are administered in the form of a tablet of solid material inserted under the skin, their physiological effectiveness is greatly increased, and their duration of action prolonged [Deanesly and Parkes, 1937]. The possibility that the subcutaneous administration of insulin in the form of tablets of solid material might result in an increased and prolonged action therefore seemed worthy of investigation.

METHODS

Animals. Rabbits weighing 1·5–2·0 kg. were used in all the experiments recorded in this paper. They received an ample diet of cabbage, oats, and hay.

Insulin. Commercial crystalline insulin containing 0·9% of zinc was kindly supplied by Dr. J. W. Trevan of The Wellcome Physiological Research Laboratory, Langley Court, Beckenham. A highly purified amorphous preparation of insulin, purified by repeated isoelectric precipitation and containing less than 0·02% of zinc was kindly provided by Dr. T. F. Dixon of Messrs. B. D. H., Ltd. Both of these preparations assayed approximately 20 units/mg.

Solid material from protamine-insulin suspension was prepared by mixing the contents of the two vials supplied as 'Insulin Retard' by Nordisk Insulinlaboratorium, Copenhagen, and centrifuging off the suspended material. The precipitate was suspended in absolute alcohol and again spun off. This process was repeated a number of times to remove the last traces of water, and the final precipitate freed from excess alcohol in a vacuum desiccator. Solid material from 'protamine-insulin with zinc' (Burroughs Wellcome, Ltd.) was prepared in a similar manner.

Tablets were made from undiluted powdered insulin in a tablet-making machine kindly loaned by Messrs. B. D. H., Ltd. Each tablet weighed about 5 mg.

In many experiments no precautions were taken to ensure the sterility of the tablets. In some instances, however, it was considered advisable to use sterile tablets. It was found that sterilization could be effected by heating in a dry state to 100° C. for 1 hour on each of three successive days. Bacteriological tests showed that complete sterility had been induced by this process. By analogy with the results of experiments on the dry heating of the urinary gonadotrophic substance [cf. Askew and Parkes, 1933] one might expect that the physiological activity of insulin would not be lost after such treatment. The results shown graphically in Fig. 1 bear this out for crystalline insulin, although there is a decided drop in activity after heat sterilization. The results with heat sterilized low-zinc insulin were variable. In two experiments no hypoglycaemic response was observed when the equivalent of 2 units of heat sterilized material was injected into a fasting rabbit. With a 3rd batch of material apparently treated in the same way, the equivalent of 2 units exerted a definite, though diminished hypoglycaemic action in the rabbit (Fig. 1). Material from the two batches showing no activity in a dose equivalent to 2 units had not lost activity entirely, as shown by the substantial hypoglycaemic action exerted by larger doses. The subcutaneous administration of a solution containing the equivalent of 100 units of heat-sterilized insulin (crystalline or low-zinc) to non-fasting rabbits was followed by a hypoglycaemic response which differed insignificantly from that following the injection of a solution of 100 units of untreated insulin.

It should be mentioned that our samples of insulin were not specially dried before heat sterilization. It is possible that if rigorously dried material had been used, no inactivation would have occurred.

Implantation of tablets. A shaved area on the flank of the rabbit was infiltrated with a 1% solution of cocaine, and in the area of skin thus anaesthetized, an incision of length about 1 cm. was made. The incised area was further treated with cocaine, and by gentle manipulation with blunt forceps a pocket was opened between the skin and the underlying tissues, and into this pocket the tablet was dropped. Care was taken to ensure that the pocket was dry and free from blood. The incision was closed with a single stitch, precautions being taken not to disturb the tablet in any way during this and subsequent procedures. It was found that the subcutaneous implantation of a tablet could be effected in this way without causing any reaction or sign of discomfort on the part of the rabbit, and without causing the blood-sugar level to rise appreciably.

In some experiments sterile tablets were implanted with aseptic pre-

cautions, but in many instances no special precautions were taken, since the experiments were of short duration.

Blood-sugar estimations were made by the Hagedorn-Jensen method, using 0·1 c.c. of blood from the ear vein.
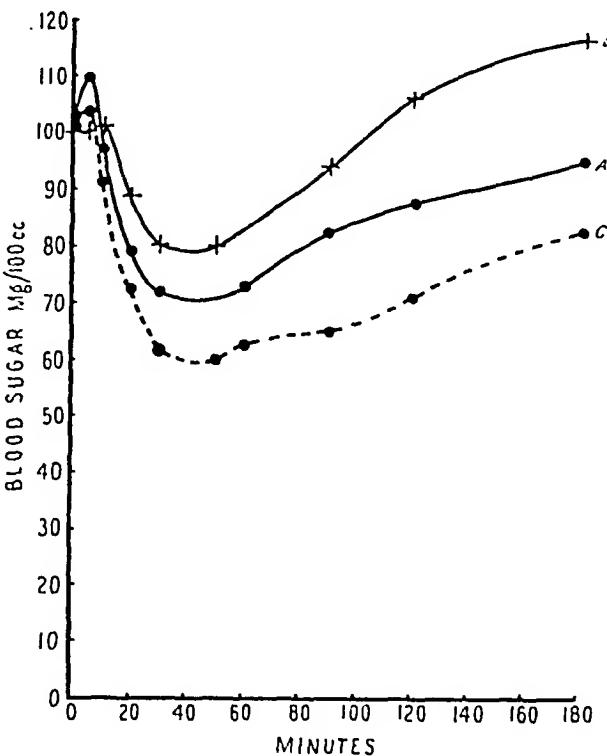
FIG. 1. The influence of dry-heat sterilization on the activity of insulin.

Curve A. Average response of 5 rabbits to the intravenous injection of the equivalent of 2 units of heat-sterilized crystalline insulin.

Curve B. Response of a rabbit to the intravenous injection of the equivalent of 2 units of heat-sterilized low-zinc insulin.

Curve C. Normal response to the intravenous injection of 2 units of insulin in the rabbit.

RESULTS

Experiments with fasting rabbits

In the first series of experiments the rabbits fasted for 16–24 hours before the implantation of the tablet, and received no food during the experiment. Eight experiments were made on the implantation of un-sterilized tablets of crystalline insulin, and 4 experiments with similar tablets of amorphous low-zinc insulin. Typical blood-sugar curves obtained from experiments of this type are shown in Fig. 2. Two experiments

were also carried out with sterile tablets of crystalline insulin, and 2 with sterile tablets of amorphous insulin. Blood-sugar curves obtained in these experiments (Fig. 2) did not differ strikingly from those with non-sterile tablets, but in all 4 experiments with sterilized tablets the animals ulti-
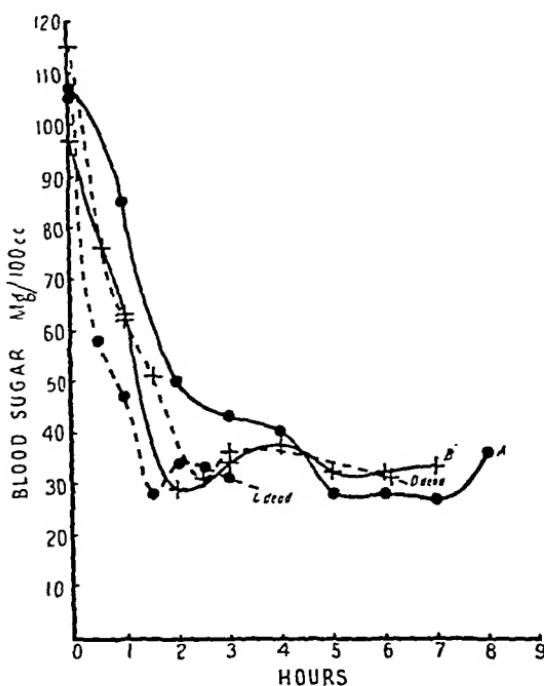
Fig. 2. The influence of the subcutaneous implantation of pellets of solid insulin containing the equivalents of 100 units, on the blood-sugar level of the fasting rabbit.

(Typical Curves)
Curve *A*. Unsterilized crystalline insulin.
Curve *B*. Unsterilized low-zinc insulin.
Curve *C*. Heat-sterilized crystalline insulin.
Curve *D*. Heat-sterilized low-zinc insulin.

mately died in hypoglycaemic convulsions, whereas only 1 death (with low-zinc insulin) occurred in the 12 experiments with unsterilized tablets. This suggested that the insulin was being absorbed more rapidly from the sterilized tablets than from those unsterilized, particularly in view of the fact that the heat-sterilized insulin was less active physiologically than the unsterilized material.

In the experiments on fasting rabbits, blood-sugar estimations were made at intervals for 7 or 8 hours after implantation of the tablet. If the animal was still alive after this period it was fed. In some instances food

was again withdrawn 16 hours later, and a series of blood-sugar estimations made. In no instance was a significant fall of blood-sugar level found during the second period of fasting, showing that the absorption
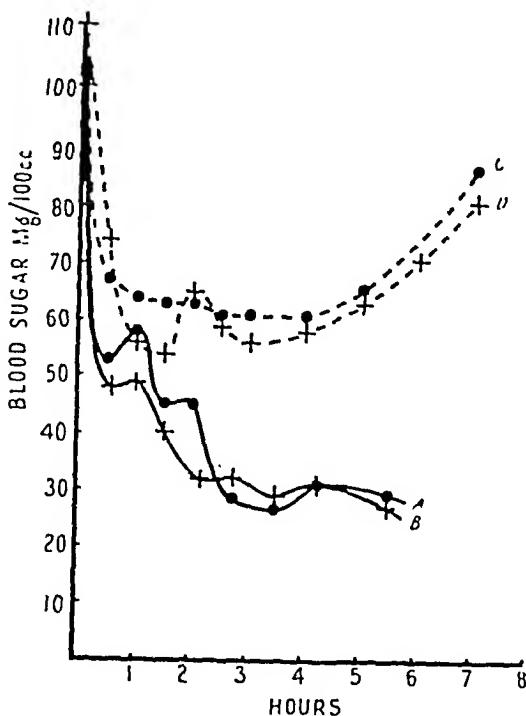
Fig. 3. The influence of the subcutaneous injection of a solution of 100 units of insulin on the blood-sugar level of the rabbit.

Curve A. Average response of 4 fasting rabbits to the subcutaneous injection of crystalline insulin.

Curve B. Average response of 3 fasting rabbits to the subcutaneous injection of low-zinc insulin.

Curve C. Average response of 3 non-fasting rabbits to the subcutaneous injection of crystalline insulin.

Curve D. Average response of 3 non-fasting rabbits to the subcutaneous injection of low-zinc insulin.

of material from the tablet was physiologically undetectable 24 hours after implantation.

Control experiments on the subcutaneous administration of a solution of insulin containing 100 units (i.e. 5 mg. of solid material) to fasting rabbits showed (Fig. 3) that both with crystalline (4 experiments) and with low-zinc insulin (3 experiments) death invariably occurred. Nevertheless, in these experiments the initial rate of fall of the blood-sugar level was not obviously greater than that observed in the experiments with subcutaneous tablets (cf. Fig. 2).

In the experiments with the low-zinc insulin, no trace of the tablet could be found 24 hours after implantation, whether sterilized or unsterilized tablets had been used. In contrast to this, post-mortem examination of the rabbits receiving either sterilized or unsterilized tablets of crystalline insulin showed the presence, at the site of implantation, of cysts about 3 mm. in diameter and 1 mm. thick. On histological examination these were found to contain clearly defined crystals. The presence of these crystals in the capsule was strikingly confirmed with the aid of the polarizing microscope (Fig. 4). In one experiment a rabbit which had received a subcutaneous implantation of a tablet of crystalline insulin, was killed 3 days after implantation, and an alkaline extract (pH 8·5) made of the tissues surrounding the point where the tablet had been implanted. The extract was cleared by centrifuging, and injected subcutaneously into another rabbit; hypoglycaemic convulsions occurred 2 hours later in the recipient rabbit, and death ensued 5 hours after injection of the extract. This showed conclusively that physiologically active insulin was present at the site of implantation 3 days after the tablet of crystalline insulin had been implanted, and there was no reason to doubt that the observed crystals were those of insulin. The formation of the capsule had apparently prevented further absorption of the material.

The formation of a capsule surrounding the crystalline insulin was not due to the presence of a non-sterile foreign body in the subcutaneous tissues, as the capsule was formed when sterile tablets of crystalline insulin were used, and was not formed when unsterilized tablets of amorphous insulin were implanted. Moreover, tablets of oestrone or testosterone may be left in the subcutaneous tissues for months without causing any sign of the reaction given by the crystalline insulin.

The subcutaneous implantation of a tablet of the solid material obtained from protamine-insulin suspension (1 experiment) and from zinc protamine-insulin (1 experiment) had no significant effect on the blood sugar level of the fasting rabbit.

Experiments with non-fasting rabbits

In the second series of experiments the rabbits did not undergo a preliminary fast, and were allowed access to food during the whole of the experimental period.

Eight experiments were carried out with unsterilized tablets of crystalline insulin, and 6 experiments with unsterilized tablets of low-zinc insulin. The average curves are given in Fig. 5. Four experiments were carried out with heat-sterilized tablets of crystalline insulin, and 4 with similarly treated tablets of low-zinc insulin. The average curves for these experiments, which are also given in Fig. 5, show that heat-sterilization exerted

no apparent influence on the effectiveness of the implanted tablets. If we take into account the diminished physiological activity of the heat-sterilized material, we must consider the possibility that insulin was being
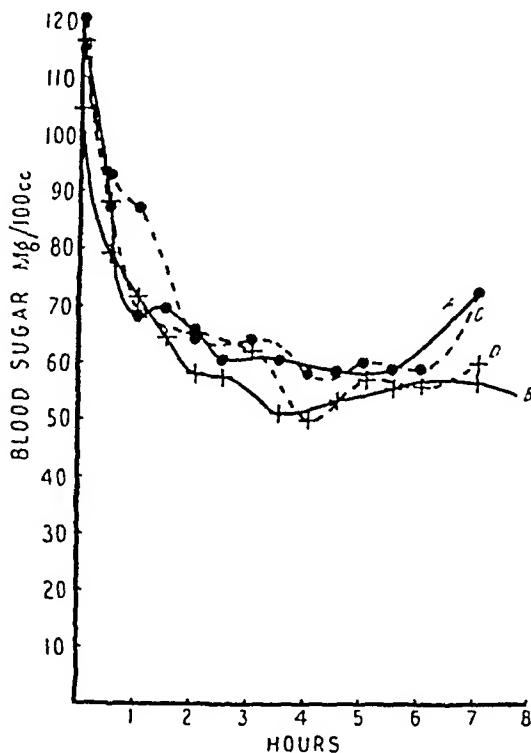
FIG. 5. The influence of the subcutaneous implantation of pellets of solid insulin containing the equivalent of 100 units, on the blood-sugar level of the non-fasting rabbit.

Curve A. Unsterilized crystalline insulin. Average curve for 6 rabbits.

Curve B. Unsterilized low-zinc insulin. Average curve for 6 rabbits.

Curve C. Heat-sterilized crystalline insulin. Average curve for 4 rabbits.

Curve D. Heat-sterilized low-zinc insulin. Average curve for 4 rabbits.

absorbed from the sterile tablets at a greater rate than from the non-sterile tablets.

The results of experiments on the influence of the subcutaneous injection of a solution of insulin containing 100 units, on the blood-sugar level of the non-fasting rabbit, are given in Fig. 3. The curves shown are those for the average response of groups of 3 rabbits receiving insulin which had not been heat-sterilized. Other results, not depicted, showed that heat-sterilization of the dry insulin exerted no appreciable influence on the hypoglycaemic action of a subcutaneously injected solution containing

the equivalent of 100 units. Comparison of the results given in Figs. 3 and 5 shows that the influence of the tablets was slightly more prolonged than that of the solutions. In both sets of experiments there were indications that the low-zinc insulin was slightly more effective than the crystalline material.

DISCUSSION

The results show clearly that the hypoglycaemic action of subcutaneously implanted tablets of insulin is only very slightly more prolonged than that of subcutaneously injected solution, when big doses are used. In this respect insulin differs from many other physiologically active substances.

The very slight difference between the results obtained with tablets of crystalline insulin and those with low-zinc insulin appears at first somewhat surprising in view of the fact that much of the crystalline material is not absorbed, whereas the tablets of low-zinc insulin completely disappear. Inactivation in the body of some of the absorbed insulin might account for these findings, but there is no reason to doubt that insulin can be excreted by the kidneys [see Hill and Howitt, 1936, for references], and it seems possible that much of the big doses used in the present series of experiments was excreted in this manner. Thus the similar results obtained with the two types of insulin would suggest that the balance between absorption and excretion (or inactivation) was similar in the two cases, the balance being slightly greater with the low-zinc material.

The formation of a capsule containing crystals of unabsorbed material when tablets of crystalline insulin were implanted might be attributed to the action of the zinc in the crystals, particularly in view of the absence of such a reaction when tablets of low-zinc insulin were implanted. Experiments on the subcutaneous implantation of zinc salts (e.g. zinc sulphate) have so far failed to produce any local reaction, absorption being complete and rapid. A combination of zinc salts and protein might, however, exert a different effect when subcutaneously implanted.

SUMMARY

1. Insulin can be sterilized in the dry state by heating to 100° C. for 1 hour on 3 successive days. Some inactivation occurred as the result of this process in our experiments, the crystalline insulin being less affected than the low-zinc material.

2. In the rabbit the hypoglycaemic action of subcutaneously implanted tablets of insulin containing approximately 100 units is only slightly more prolonged than that of 100 units of subcutaneously administered solution. Heat-sterilized tablets exerted an action similar to that of unsterilized tablets.

3. Tablets of low-zinc insulin had been completely absorbed within 24 hours of implantation. With crystalline material (sterilized or un-sterilized) a capsule was formed at the site of implantation, containing well-defined crystals of insulin. In spite of this the effectiveness of the crystalline tablets was very little different from that of the tablets of low-zinc insulin. This suggests that part of the absorbed insulin had been inactivated or excreted.

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Fig. 4 *a*. Histological section of cyst found four days after implantation of a 7 mg. tablet of zinc crystalline insulin. Usual histological technique except for omission of differentiation in acid alcohol. × 67
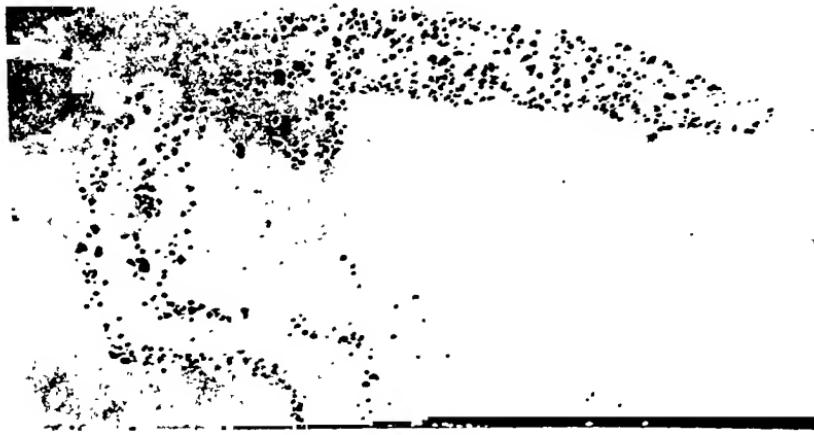
Fig. 4 *b*. Same section under polarized light, showing presence of abundant crystals and restriction of them to the walls of the cyst. The two odd crystals in the top left-hand corner have been washed away from the tissue during staining and mounting. × 67

3. Tablets of low-zinc insulin had been completely absorbed within 24 hours of implantation. With crystalline material (sterilized or unsterilized) a capsule was formed at the site of implantation, containing well-defined crystals of insulin. In spite of this the effectiveness of the crystalline tablets was very little different from that of the tablets of low-zinc insulin. This suggests that part of the absorbed insulin had been inactivated or excreted.

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SEPARATION OF FOLLICLE-STIMULATING FRAC-
TION FROM PITUITARY GONADOTROPHIN

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(*Received 17 May 1939*)

In 1930 Fevold and Hisaw reported a separation of pituitary gonado-
trophin into two fractions—a follicle-stimulating hormone (FSH) and a
luteinizing hormone (LH). FSH when injected *into immature* or hypo-
physectomized rats caused follicular growth in the ovaries with no
luteinization. LH injected by itself had no follicle-stimulating properties
in such animals but would luteinize the follicles produced by FSH in-
jection. Their separation [Fevold, Hisaw, and Leonard, 1931] depended
on the greater solubility of FSH in cold distilled water. Evans, Meyer,
and Simpson [1933] were unable to confirm these results, nor were Van
Dyke and Wallen-Lawrence [1933].

In 1933 Fevold, Hisaw, Hellbaum, and Hertz published a method of
separation by the selective adsorption of LH on benzoic acid. Saunders
and Cole [1938] failed to confirm this, though they obtained some degree
of separation.

In 1934 Wallen-Lawrence separated two fractions by precipitation with
alcohol at 6° C. The precipitate with 40% alcohol was predominantly
luteinizing, that with 55% alcohol purely follicle stimulating. Saunders
and Cole [1938] again failed to confirm this method of separation.

Leonard and Smith [1933] reported that when extracts of castrate and
pregnancy urine were injected into the same animal, the resulting increase
in ovary weight was more than the added increase in weight of the four
ovaries obtained after injecting castrate urine extract into one animal and
pregnancy urine extract into another. Evans, Simpson, and Austin [1933]
found the same augmentation when pregnancy urine was given with
anterior pituitary extracts and claimed to have isolated a separate
'synergist' from the latter. When Fevold and Hisaw [1934] *repeated*
this work the synergist they obtained was predominantly follicle stimulat-
ing and they concluded that the augmentation was due to the combined
action of FSH and LH on the ovary.

Evans, Simpson, Korpi, and Wonder [1936] separated three fractions
from anterior lobe extracts by fractional precipitation with different
saturations of ammonium sulphate. While their LH fraction was con-

I

taminated with one of the other two fractions, they claimed to obtain a pure FSH fraction and a fraction (ICSH = interstitial cell stimulating hormone) which had no effect on the immature female rat but repaired the atrophic interstitial cells in the gonads of hypophysectomized rats of both sexes. Their LH when contaminated with FSH did not have this effect [Evans, Simpson, Pencharz, 1937] and so differed from the LH principle of Fevold and collaborators [Greep, 1936; Greep and Fevold, 1937]. The principle differentiation of the ICSH from Fevold's LH principle and pregnancy urine was its failure to give augmentation when injected in combination with FSH. In 1937 Fevold repeated this work and was unable to obtain any evidence of a separate ICSH fraction. At the same time he published a new method for separating the fractions, based on ammonium sulphate precipitation.

Our own attempts at separation of acetone dried horse pituitary gland reported below lead us to conclude that the methods of separating FSH and LH so far advanced are not reproducible, owing to interference by the large amount of inert material present in the extracts. All our methods are based on ammonium sulphate precipitation, but are applied to extracts of different degrees of purity.

BIOASSAYS

Immature female rats were injected daily with the various fractions for a period of 5 days. The dosage figures given throughout this paper are expressed as the total dose given in the five injections in terms of the weight of original dried gland. In some cases hypophysectomized immature female rats were used so that secretion by the injected animal's own pituitary should not interfere with the assay. Hypophysectomized adult male rats were occasionally used for the assay of suspected ICSH activity. The hypophysectomy was performed from the retro-pharyngeal approach. The females were killed on the 6th day, the ovaries and uteri dissected out and fixed in Bouin's solution. When in the 70% alcohol stage of the dehydration process the ovaries were roughly dried on filter-paper and weighed. Serial sections were prepared from most of the ovaries for histological examination. The degree of luteinization and follicle stimulation given in the tables was based on this examination. The male hypophysectomized rats were injected daily for 7 days, killed on the 8th when the testes, prostate, and seminal vesicles were dissected out and subjected to the same procedures for weighing and histology as the ovaries and uteri of the females. As the object of this work was mainly of a qualitative nature, groups of 3 animals for the bioassays were considered to be sufficient.

Preparation of crude extract

Evans *et al.* [1936], using fresh sheep pituitary gland as starting material, extracted with Ba(OH)$_2$ or NaOH solutions. These solutions were not very effective for the extraction of the gonadotrophic activity from acetone dried horse pituitary gland, so a glycine-NaOH-NaCl buffer solution at pH 10 was used as extracting agent (mixture of 4 parts of N/10 NaOH and 6 parts of a solution of 7·505 g. glycine and 5·85 g. NaCl per litre). The dried pituitary gland was ground with sand in a mortar and the buffer solution added. The extraction was carried out at 0° C. overnight, the mixture being mechanically stirred throughout. The residue was centrifuged off. In most cases a fresh extract was used for each attempted separation of the fractions. It was found that the recovery of activity was more complete when a smaller initial weight of dried gland was used. This was presumably due to a more effective grinding in the mortar with small quantities.

Fractionation of Crude Buffer Extracts

In brief, Evans *et al.* [1936] using their procedure I found that ICSH activity was precipitated by 40% saturation with ammonium sulphate (0·4 SAS) at pH 7. Raising the degree of saturation in the supernatant fluid to 60% (still at pH 7) precipitated the LH activity. Following the removal of this precipitate, the supernatant fluid was raised to 80–90% saturation with ammonium sulphate at pH 5 and the FSH activity precipitated.

Process I

We repeated this procedure using 175 c.c. of crude buffer extract (= 5 g. dried gland), but were unable to confirm their results. The 0·4 SAS precipitate contained both FSH and LH activity even after three reprecipitations from 0·4 SAS. The 0·6 SAS precipitate contained practically pure FSH activity, while the 0·9 SAS precipitate had no detectable gonadotrophic activity. Thus in our experiments both FSH and LH activity were precipitated at lower degrees of ammonium sulphate saturation than in the experiments of Evans *et al.* [1936].

Process II

These results and the description by Evans, *et al.* [1937] of an improved method for the separation of the ICSH fraction, suggested the modification of the previous process outlined in Table I.

Whereas in Process I the pH of the precipitations varied, in this proce[
they were all carried out at pH 5. The biological assays of the variou
fractions are given in Table II.

Table I. *Fractionation of gonadotrophin*

Extract (HP11)
equivalent to 4 g. HP in 140 c.c.
made up to 0·2 SAS at pH 5.

Precipitate washed
twice with 0·2 SAS
solution at pH 5
(HP30a)

Supernatant
made up to 0·3 SAS
at pH 5

Supernatant
made up to 0·5 SAS
at pH 5.

Precipitate
washed twice with
0·3 SAS solution
at pH 5.
(HP31a)

Precipitate
reprecipitated
from 0·5 SAS at
pH 5, three times
from successive
smaller volumes
(HP33a)

Supernatant
made up to 0·9 SAS
at pH 5

Supernatant
discarded

Precipitate
redissolved in
9 c.c. Made up to
0·5 SAS at pH 5

Supernatant
made up to 0·9 SAS
at pH 5

Precipitate
recombined
with 0·5 SAS
fraction

Precipitate
redissolved in
3 c.c. made up to
0·4 SAS at pH 4·5

Supernatant
discarded

Precipitate

Supernatant made up to 0·9 SAS

Precipitate (HP32a)

Supernatant
discarded

The 0·2 SAS precipitate (HP30a) contained no gonadotrophin, even
though tested on male hypophysectomized rats for ICSH activity. The
0·3 SAS precipitate (HP31a) contained both FSH and LH, but a dose of
1·3 g. was necessary to stimulate the ovaries of injected rats. Most of the

LH seemed to be precipitated with 0·5 SAS, though this precipitate (HP33a) still contained FSH after three reprecipitations. The 0·9 SAS precipitate (HP32a) was dissolved and further precipitates removed at 0·5 SAS and 0·4 SAS, as shown in Table I. The final precipitate when tested was predominantly follicle stimulating, though producing a few small corpora lutea and some interstitial cell repair when injected into hypophysectomized rats.

Table II. *Effects of gonadotrophic fractions obtained by Process II on immature female rats*

		Biological Assays			Histological Examinations	
No.	Preparation	Dose, equivalent of mg. HP powder	Ovaries mg.	Uterus mg.	FSH	LH
HP11	Crude extract	10	82	133	+++	+
HP30a	0·2 SAS precipitate	1300	5 H	25	—	—
HP31a	0·3 SAS precipitate	1300	22	155	++	+
HP33a	0·5 SAS precipitate	400	144	155	+++	+++
HP32a	0·9 SAS precipitate	600	48 H	122	+++	(+)
Control immature			14	33	..	..
Control hypophysectomized			6	20	..	..

H = hypophysectomized.

This process therefore yielded a relatively pure FSH fraction. It was not, however, quantitatively satisfactory since there had been a loss of FSH in both the 0·3 and 0·5 SAS precipitates. This second failure to repeat the work of Evans *et al.* suggested that the differences might be due to the different starting materials. As the presence of FSH in the precipitates at lower degrees of ammonium sulphate saturation might be due to adsorption on to the inert material also precipitated, we decided to attempt the separation of the fractions from a purer starting material.

Fractionation of sulphosalicylic acid soluble material

The addition of sulphosalicylic acid to pregnant mare serum, bringing the pH to 2, precipitates 98% of the inert material, leaving the gonadotrophic activity in the supernatant fluid [Rinderknecht, Noble, and Williams, 1939]. Crude buffer extracts of the dried horse gland were similarly treated. It was found that the supernatant fluid contained only about half of the gonadotrophic activity. The precipitate contained some of the activity but much was destroyed. (The loss was not due to separation of the FSH and LH with consequent loss of augmentation, since the

original activity was not recovered when precipitate and supernatant were combined and injected.)

Process III

A buffer extract of 10 g. of dried gland was concentrated to 83 c.c. This extract was then brought to pH 2 by the addition of 33% sulphosalicylic acid and the resulting precipitate removed. Ammonium sulphate was added to the supernatant fluid until it was 45% saturated. A precipitate was removed and the supernatant fluid raised to 0·9 SAS. The 0·9 SAS precipitate was redissolved and precipitates were taken at 0·45 SAS and 0·9 SAS as before. The second 0·9 SAS precipitate was redissolved and similarly treated. The pH was kept as 7 throughout these procedures. The third and final 0·9 SAS precipitate had no detectable gonadotrophic activity. The final 0·45 SAS precipitate contained FSH.

Process IV

Since the FSH had been taken down with the 0·45 SAS precipitates in Process III, that process was repeated, removing precipitates at 0·4 SAS instead of 0·45 SAS. The starting material was less concentrated. Ten grammes of dried gland was extracted with 280 c.c. buffer solution and 100 c.c. of this solution treated with sulphosalicylic acid. There was no improvement in the final results, since FSH was still taken down with the precipitates at the lower degree of ammonium sulphate saturation.

Process V

One hundred cubic centimetres of the buffer extract used in Process IV were brought to pH 2 with 33% sulphosalicylic acid and the supernatant fluid raised to only 0·35 SAS and adjusted to pH 7 before removal of the first precipitate. This precipitate contained little FSH. Injected in a dose (200 mg.) 10 times greater than the minimal effective dose of the sulphosalicylic acid soluble material, it produced ovaries of 20 mg. The supernatant fluid was raised to 0·9 SAS and the precipitate redissolved. When this solution was raised to 0·35 SAS no precipitate was formed until the pH was adjusted to 4·5. This precipitate contained more FSH. When injected in the same dose of 200 mg., ovaries of 50 mg. were produced. The supernatant fluid was raised to 0·9 SAS and the precipitate when injected in a 200 mg. dose produced ovaries of 40 mg. in the hypophysectomized rat. These ovaries showed that follicular growth and interstitial cell proliferation had been stimulated. Fragmentation of the granulosa in some follicles showed that traces of LH were still present in this fraction. This process was repeated on the remaining crude buffer extract omitting
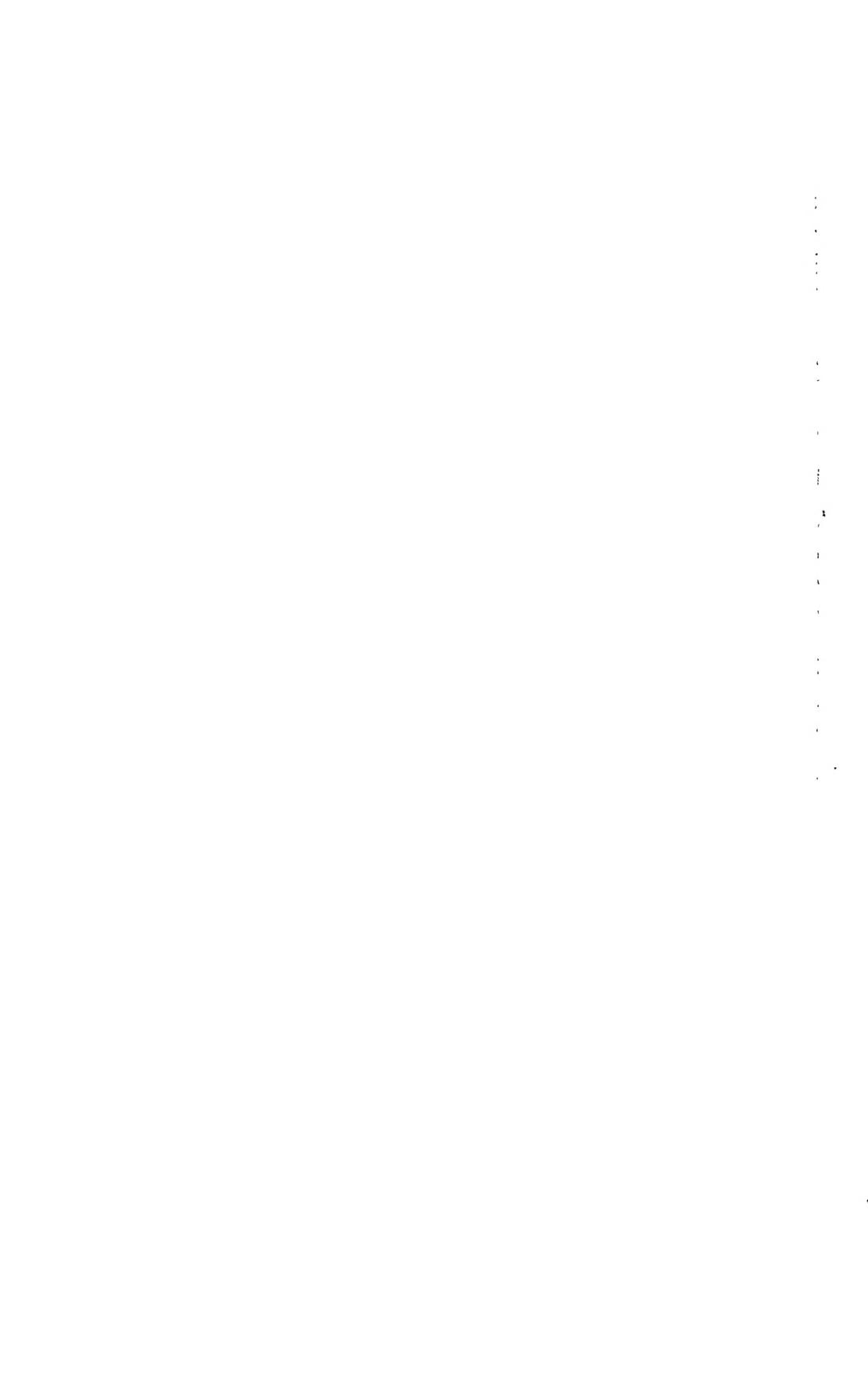
Fig. 1. Ovary of hypophysectomized rat 744. Received no injection. Wt. of single ovary 3 mg. × 20.

Fig. 2. Ovary of hypophysectomized rat 871. Received tion of crude extract HP541. Wt. of single ovary 26 mg. × 20.
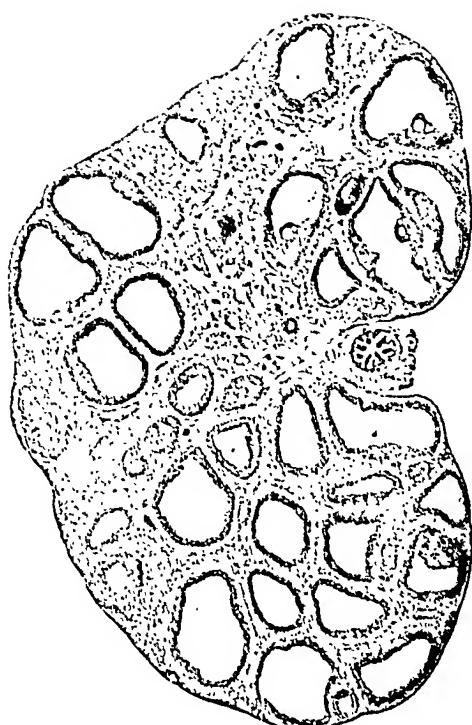
Fig. 3. Ovary of hypophysectomized rat 895. Received FSH extract HP5510. Wt. of single ovary 21 mg. × 20.

the sulphosalicylic acid preparation. Both 0·35 SAS precipitations were made at pH 4·5. The final 0·9 SAS precipitate had to be injected in a dose of 1000 mg. to produce any ovarian response. The preliminary sulphosalicylic acid precipitation therefore increased the yield of FSH in the final 0·9 SAS precipitate. The yield obtained by the whole process including the sulphosalicylic acid treatment was also an improvement on the yield obtained from the crude extract by Process II (HP32a, Table II). The effective dose was one-third of that of HP32a.

These results indicated that a preliminary step in the purification of the gonadotrophin increased the efficiency of the subsequent separation of FSH and LH, although the actual purification process used in this instance was quantitatively unsatisfactory.

Fractionation of 50% Alcohol Soluble Material

When an equal volume of absolute alcohol was added to a crude buffer extract the precipitate contained no gonadotrophic activity. The supernatant fluid was used as the starting material for another attempt to separate the two fractions.

Process VI

The details of this process are given in Table III.

In Table IV are given the results of the biological tests.

Since previous results showed that FSH was taken down by 0·35 SAS precipitations at pH 4·5, the first two 0·35 SAS precipitations in this process were made at pH 7. The 0·9 SAS precipitate obtained after this procedure (HP551)' still contained much LH activity. The tables show that two further precipitates were removed at 0·35 SAS pH 4·5, and one at 0·4 SAS pH 4·5, before the last traces of LH disappeared from the 0·9 SAS precipitable material. There was some loss of FSH in these 0·35 SAS precipitates (e.g. HP555). The final 0·9 SAS precipitate (HP5510) was the purest follicle-stimulating fraction we obtained. Injected in a dose of 450 mg. into two hypophysectomized rats, it produced ovaries of 42 mg. These ovaries contained no corpora lutea and only occasional traces of granulosa fragmentation and thecal luteinization. The stromal elements in the ovaries were obviously stimulated. A typical ovary obtained after injection of this fraction is shown in Fig. 3, compared with the ovary from a control hypophysectomized rat (Fig. 1) and from one injected with a crude buffer extract (Fig. 2). Comparison of the effective dose of this FSH fraction with that obtained from the sulphosalicylic acid soluble material (Process V) shows that the yield is not significantly improved.

Table III. *Fractionation of gonadotrophin*

25 c.c. extract HP461, equivalent to 1000 mg. HP,
precipitated with 50% alcohol at pH 7

Precipitate
discarded
(HP55)

Supernatant
brought to 75% alcohol
at pH 5

Precipitate
redissolved in 5 c.c.
of water. Made up to 0·35 SAS at pH 7

Supernatant
discarded

Precipitate
reprecipitated at 0·35 SAS
from 2 c.c. Supernatant pooled
with first 0·35 SAS supernatant

Supernatant
made up to 0·9 SAS at pH 5

Precipitate
redissolved in 3 c.c.
Made up to 0·35 SAS at pH 7

Supernatant
discarded

Supernatant, made up to
0·9 SAS at pH 5

Precipitate

Precipitate
(HP551) redissolved in 2 c.c. made up to 0·35 SAS at pH 4·5

Precipitate

Supernatant
made up to 0·9 SAS at pH 4·5

Precipitate (HP552)
Redissolved in 1 c.c. made
up to 0·35 SAS at pH 4·5

Supernatant
discarded

Precipitate
(HP555)

Supernatant
made up to 0·9 SAS

Precipitate (HP556)
Redissolved in 0·5 c.c. made
up to 0·4 SAS at pH 4·5

Supernatant
discarded

Precipitate (HP557)

Supernatant
made up to 0·9 SAS

Supernatant
discarded

Precipitate
(HP5510)

Fractionation of Ammoniacal Extract

Process VII

The method of Fevold [1937], like those of Evans and his colleagues, depends on the greater solubility of FSH in certain saturations of ammonium sulphate, but also makes use of his finding that LH shows a minimum solubility at pH 4–4·2. We repeated this method, extracting 10 g. of dried horse pituitary gland with 500 c.c. of 0·02 N ammonium hydroxide. This extracting agent, found by Fevold to be effective

Table IV. *Effects of gonadotrophic fractions obtained by Process VI on immature female rats*

| No. | Preparation | Biological Assays | | | Histological Examinations | |
		Dose, equivalent of mg. HP powder	Ovaries mg.	Uterus mg.	FSH	LH
HP461	Crude extract	10	48	106	+++	++
HP55	50% alcohol precipitate	200	12	43	−	−
HP551	0·9 SAS precipitate	150	72	120	+++	++
HP552	0·9 SAS precipitate	150	62	131	+++	++
HP555	0·35 SAS precipitate	125	52	80	+++	++
HP556	0·9 SAS precipitate	250	40	118	++	++
HP559	0·4 SAS precipitate	250	12	49	+	−
HP5510	0·9 SAS precipitate	450	42 H	82	+++	−
Control immature	. . . . . .		14	33	..	..
Control hypophysectomized	. . . .		6	20	..	..

H = hypophysectomized.

when applied to acetone dried sheep gland, was not very effective when applied to dried horse gland. Although the subsequent fractionation method was followed exactly, the fraction which, according to Fevold, should be free from LH gave the same ovarian response as the crude ammoniacal extract. The 0·2 SAS precipitates, which should have purely LH activity, were found to contain much FSH in our experiment. Eggleton and Robson [1939] have repeated this method of separation, also using dried horse gland as starting material, without success.

DISCUSSION

It has been pointed out that although many workers have reported a separation of the FSH and LH present in pituitary gonadotrophin, no single method has ever been successfully confirmed. Our own findings point to the existence of two separate fractions, but the details of the

Table III. *Fractionation of gonadotrophin*

25 c.c. extract HP461, equivalent to 1000 mg. HP,
precipitated with 50% alcohol at pH 7

Precipitate
discarded
(HP55)

Supernatant
brought to 75% alcohol
at pH 5

Precipitate
redissolved in 5 c.c.
of water. Made up to 0·35 SAS at pH 7

Supernatant
discarded

Precipitate
reprecipitated at 0·35 SAS
from 2 c.c. Supernatant pooled
with first 0·35 SAS supernatant

Supernatant
made up to 0·9 SAS at pH 5

Precipitate
redissolved in 3 c.c.
Made up to 0·35 SAS at pH 7

Supernatant
discarded

Supernatant, made up to
0·9 SAS at pH 5

Precipitate

Precipitate
(HP551) redissolved in 2 c.c. made up to 0·35 SAS at pH 4·5

Precipitate

Supernatant
made up to 0·9 SAS at pH 4·5

Precipitate (HP552)
Redissolved in 1 c.c. made
up to 0·35 SAS at pH 4·5

Supernatant
discarded

Precipitate
(HP555)

Supernatant
made up to 0·9 SAS

Precipitate (HP556)
Redissolved in 0·5 c.c. made
up to 0·4 SAS at pH 4·5

Supernatant
discarded

Precipitate (HP557)

Supernatant
made up to 0·9 SAS

Supernatant
discarded

Precipitate
(HP5510)

SUMMARY

1. Attempts to separate a pure follicle-stimulating fraction from horse pituitary extracts by the methods of Evans *et al.* [1936] and of Fevold [1937] were unsuccessful.

2. By modifications of these procedures a relatively pure follicle-stimulating fraction was obtained.

3. Removal of inert material from the crude pituitary gland extracts improved the results.

4. It is suggested that the inert materials present in crude extracts seriously interfere with the separation of LH and FSH.

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separation procedures adopted by Evans *et al.* [1936] and by Fevold [1937] had to be modified to bring about a satisfactory separation. We obtained no evidence of the existence of a separate ICSH fraction. We always found stromal proliferation associated with our purest FSH fractions (cf. Fig. 3). This finding is in direct conflict with the finding of Evans *et al.* [1936] that the ICSH fraction was precipitated at lower concentrations of ammonium sulphate than the LH fraction. In human pituitary glands having no luteinizing activity, ICSH is associated with the FSH principle [Witschi, 1937; Noble, Rowlands, Warwick, and Williams, 1939].

Our failure to confirm the separation methods of Evans *et al.* and Fevold, and the failure of Eggleton and Robson to confirm the method of Fevold, may be due to the use of horse pituitary gland instead of sheep pituitary gland which has been used by all previous workers in this field. Apart from the comparative failure of extracting solutions which are effective in the extraction of gonadotrophic activity from sheep pituitary gland, when applied to horse gland, we have other evidence that species difference alters the behaviour of pituitary gonadotrophin. Thus we found that the gonadotrophin present in acetone dried horse pituitary extract is almost completely precipitated by flavianic acid, while that present in a similar acetone dried ox gland extract is not so precipitated. Unless one postulates a chemical difference in the gonadotrophin present in different species, this implies that the chemical behaviour of the hormones is altered by the presence of the inert material in the gland. The hormones may be bound up to inert protein molecules, as suggested by Collip [1937], or the inert material may affect the behaviour of the hormones in some other way. These inert materials may then differ from species to species, and thus explain the species difference in the chemical behaviour of gonadotrophin.

Our finding that some degree of purification of the pituitary gonadotrophin previous to the separation process improves the yield of FSH in the purest fraction suggests that the inert material may also interfere with the separation process. If this is so, the failure of other workers using the same starting material to repeat the various reported separation procedures may be explained. Even slight differences in such things as the condition of the gland used as starting material, the method of extraction and the carrying out of every subsequent procedure might affect the chemical nature of the inert material. Since this material is present in such preponderate amounts, slight changes in its nature may seriously affect the behaviour of the hormones. This suggests that easily reproducible results will not be obtained until a separation procedure is applied to a much purer gonadotrophic extract than has been used hitherto.

organs were dissected out and fixed in Bouin's solution, and weighed from 70% alcohol.

In the experiments to determine the effects on lactation the mother of the litter was given the stilboestrol solution to drink, commencing the day after delivery. In every case the number of rats in the litter was limited to 5, and the litters were weighed at regular intervals. In some instances the expectant rat was started on aqueous stilboestrol 3–4 days before the expected date of parturition.

Aqueous solutions of stilboestrol were prepared by Mr. Fitzgerald. The procedure adopted was to dissolve 10 mg. of stilboestrol in a few c.c. of absolute alcohol. This was stirred into 2 litres of distilled water. Following 24 hours in a mechanical shaker, it was found that the oestrogen remained in solution. This solution, containing 5 μg. per c.c., was diluted with distilled water if weaker solutions were required. In order to check approximately the amount of stilboestrol in weak solutions they were assayed for oestrogen activity on ovariectomized adult rats. Experiments are in progress to evolve a chemical method of assay for weak solutions.

RESULTS

Adult Rats

In the initial experiment two rats were placed in the same cage and the total daily amount of stilboestrol solution consumed measured for the two animals. Eleven pairs of male rats were given solutions to drink containing stilboestrol in a concentration of from 0·1 μg. to 5·0 μg. per c.c. Three pairs of control animals were allowed distilled water to drink. The animals were killed after 28 days. The results obtained for these rats are given in Table I.

A similar experiment was followed using female rats. Nine pairs of animals were given solutions of stilboestrol containing from 0·05 μg. to 5·0 μg. per c.c. These results are shown in Table II.

From the data obtained for the adult male and female animals it may be seen that this form of stilboestrol administration was quite effective. Inhibition of body-growth was produced and the inhibition of the secretion of gonadotrophic hormone was reflected by a lowered weight of the testes, and in some cases of the ovaries. The seminal vesicles and prostate were atrophied. Enlargement of the adrenal glands and the uterus was observed. In the male animals all cases tested to as low as 2·4 μg. per day would appear to reduce body-growth. When a level of 15·9 μg. per day was reached the effect was very marked. The weights of the gonads showed a wide variation, but in some cases normal gonads were present in animals where growth was retarded. In the female rats doses below 1·9 μg. per day apparently had little effect. With larger doses growth was

EFFECTS OF CONTINUOUS ORAL ADMINISTRATION OF AQUEOUS DIETHYLSTILBOESTROL SOLUTIONS TO RATS

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FOLLOWING the prolonged administration of synthetic oestrogens to rats it has been shown that changes occur which are associated with a decreased function of the anterior pituitary gland [Noble, 1938a, 1938b, 1939]. When crystals or tablets of active substances were implanted into the subcutaneous tissues it was found that although they produced the typical changes the amount absorbed was very great over a prolonged period. Since inhibition of body-growth, lactation, and gonadotrophic hormone secretion of the anterior pituitary gland is readily produced by oestrogens, it was thought to be of importance to determine quantitatively the amount of oestrogen necessary to produce these effects. The relatively high oestrogenic activity of diethylstilboestrol when administered orally [Dodds, Lawson, and Noble, 1938; Parkes, Dodds, and Noble, 1938; Emmens, 1939], and its comparatively high solubility in aqueous solution, suggested that the oral administration of aqueous solutions could be adopted. By the substitution of stilboestrol solutions of varying concentrations in place of the animal's drinking water, it has been possible to administer graded doses of this oestrogen over prolonged periods. Such a method of administration has obvious advantages, and ensures a more or less continuous absorption of the substance.

METHODS

Rats of an original Wistar strain raised in this Institute have been used throughout. During the experiment they were fed exclusively on a dry food compressed in cakes, of a similar type to that described by Thomson [1936]. The fluid intake was measured over a preliminary control period, the animals being allowed water to drink. The oestrogen solution was then substituted in place of drinking water for 28 days, and the amount consumed measured daily. In some cases the animals were placed in metabolism cages and the urine excretion also measured daily. The rats were maintained as far as possible at a constant temperature throughout the experiment. They were weighed at regular intervals, and the body-weight has been used as an index of body-growth. At autopsy the various

The wide variation in response of the two animals in the same cage suggested that the individual intake of stilboestrol solution of the two animals varied greatly. It appeared essential, therefore, to repeat the experiment with only one rat in each cage. The response of male animals to four different concentrations of stilboestrol solution (1·6 μg., 0·8 μg., ·4 μg., and 0·1 μg. per c.c.) was determined. The amount of stilboestrol consumed daily, the daily fluid intake, and alteration in body-weight have been calculated as the average per rat for each group of three or four

Table II. *The effect of oral administration of stilboestrol to female adult rats for 28 days*

Stilboestrol		Initial weight g.	Weight change g.	Adrenals mg.	Pituitary mg.	Ovaries mg.	Uterus mg.
μg. per c.c.	Daily dose μg.						
0·0	0·0	121	+35	37	6·0	45	301
		133	+24	37	6·0	45	424
0·05	0·9	159	+30	48	5·5	45	358
		139	+36	56	5·0	52	285
0·05	0·9	162	+34	58	7·0	47	290
		146	+35	54	5·5	59	382
0·1	1·9	155	+ 9	42	7·0	60	394
		140	+30	53	6·5	48	549
0·1	2·2	159	+11	43	10·0	55	421
		153	+ 5	28	10·0	26	395
0·4	3·6	150	+ 6	44	9·5	42	398
		140	+16	54	8·0	47	350
0·4	3·8	144	+ 7	37	6·5	44	361
		138	+ 6	38	7·0	34	671
5·0	40·5	150	− 2	60	6·0	27	385
		158	−36	51	6·5	19	407
5·0	68·0	153	− 1	66	9·5	36	480
		128	+ 8	62	14·0	20	535
5·0	68·5	149	−11	60	7·0	25	373
		146	+14	50	7·0	52	622

Table III. *The effect of oral administration of stilboestrol on fluid intake*

Number of rats.	Stilboestrol μg. per c.c.	Fluid intake (c.c. per rat per day)		
		Preliminary period. Water	Stilboestrol solution	Average decrease in fluid intake
8	0·1	28·5	23·8	4·7
1	0·2	32·6	24·2	8·4
4	0·3	29·2	19·1	10·0
8	0·4	26·2	13·7	12·5
10	5·0	25·5	12·5	13·0

affected and slight pituitary enlargement was found. Occasionally animals which were resistant to treatment increased in weight, although the oestrogen dose was large.

It was noticed that when the animals described above were transferred from the preliminary control period on water to the stilboestrol solution

Table I. *The effect of oral administration of stilboestrol to male adult rats for 28 days*

Stilboestrol		Initial weight g.	Weight change g.	Adrenals mg.	Pituitary mg.	Seminal vesicles mg.	Prostate mg.	Testes mg.
μg. per c.c.	Daily dose μg.							
0	0	151	+62	22	5·0	465	470	2259
0	0	156	+74	31	6·5	490	514	2217
0	0	150	+75	32	5·5	523	584	2309
0	0	147	+85	35	7·0	265	407	2116
0	0	154	+68	25	5·0	465	483	2514
0	0	146	+77	34	5·5	525	472	2165
0·1	2·4	168	+39	26	6·0	397	468	2330
		163	+36	43	7·0	315	475	2196
0·1	3·0	170	+36	38	6·0	61	190	1745
		160	+59	32	5·0	239	367	2012
0·2	4·8	164	+15	32	7·0	156	265	1726
		176	+34	42	7·0	152	265	1975
0·2	4·9	168	+28	29	6·0	48	91	581
		164	+37	35	7·0	247	310	602
0·3	5·7	160	+48	31	5·5	423	472	2537
		166	+17	42	6·0	29	62	1024
0·3	5·8	174	+34	27	6·0	357	367	2275
		167	+30	32	7·0	204	279	2240
0·4	6·5	160	+44	34	5·5	113	227	1744
		164	+26	35	7·0	118	265	2136
0·4	8·1	157	+31	33	7·0	30	70	524
		166	+33	27	6·0	527	657	2320
1·0	15·9	155	−14	25	7·0	35	75	547
		156	−6	38	6·0	18	44	333
5·0	44·8	152	−23	25	4·5	21	57	263
		152	−17	37	5·0	20	50	266
5·0	93·7	158	−9	45	9·0	35	80	384
		159	−5	55	8·0	41	90	477

the volume of fluid consumed was always reduced. Also, the amount by which the fluid intake was decreased appeared to be related to the concentration of the stilboestrol solution. The average amount of water consumed over a control period of 4–7 days may be compared with the average amount of stilboestrol solution, of different concentrations, consumed during the experimental period in Table III.

Table IV. *The effect of oral administration of stilboestrol to individual male adult rats for 28 days*

Stilboestrol		Initial weight g.	Weight change g.	Adrenals mg.	Pituitary mg.	Seminal vesicles mg.	Prostate mg.	Testes mg.
µg. per c.c.	Daily dose µg.							
0·1	2·4	163	+62	30	7·0	580	556	2104
0·1	2·4	165	+68	41	8·0	200	403	2635
0·1	2·7	160	+44	38	8·0	300	447	2287
0·4	5·7	160	+36	31	6·0	341	481	2177
0·4	7·6	188	+51	34	6·5	383	485	2320
0·4	8·0	174	+38	32	6·0	440	495	2237
0·8	10·4	164	+15	28	6·0	94	187	2078
0·8	11·5	164	+23	35	7 0	110	243	2224
0·8	12·6	171	+6	42	6·5	43	90	926
0·8	12·8	157	+26	48	7·0	45	139	1590
1·6	12·8	176	− 7	30	5·0	40	90	1293
1·6	14·4	174	0	35	7·0	35	106	1932
1·6	19·2	175	+15	38	6·5	167	275	2280
1·6	19·5	172	+11	34	7·0	42	90	531

Table V. *The effect of oral administration of stilboestrol to male adult rats for 28 days*

Number of rats	Stilboestrol µg. per c.c.	For weeks	Average per rat		
			Daily dose µg.	Fluid per day c.c.	Weight change g.
6	0	0–1	0	27·3	+20·4
		1–2	0	28·0	+19·1
		2–3	0	29·5	+18·2
		3–4	0	30·0	+16·8
3	0·1	0–1	0	27·6	+31·0
		1–2	2·6	26·5	+19·7
		2–3	2·5	25·4	+14·7
		3–4	2·5	25·6	+10·6
		4–5	2·3	23·8	+13·0
4	0·4	0–1	0	24·0	+32·4
		1–2	7·0	17·5	+ 9·0
		2–3	7·0	17·7	+11·5
		3–4	6·9	17·4	+10·5
		4–5	7·0	17·7	+10·5
4	0·8	0–1	0	21·5	+24·5
		1–2	12·4	15·5	− 1·0
		2–3	12·0	15·0	+ 5·0
		3–4	12·0	15·0	+ 6·5
		4–5	10·9	13·7	+ 7·0
4	1·6	0–1	0	24·5	+35·4
		1–2	17·9	11·2	−10·2
		2–3	15·6	9·8	+ 1·0
		3–4	15·5	9·7	+ 8·0
		4–5	16·6	10·4	+ 6·0

rats on the four different solutions. The figures obtained during each week of treatment are shown in Table IV. The preliminary control week on water and the results on control animals given water throughout are also included.

The data obtained after autopsy on these rats is shown in Table V.

These results, while similar to those obtained in the preliminary experi-
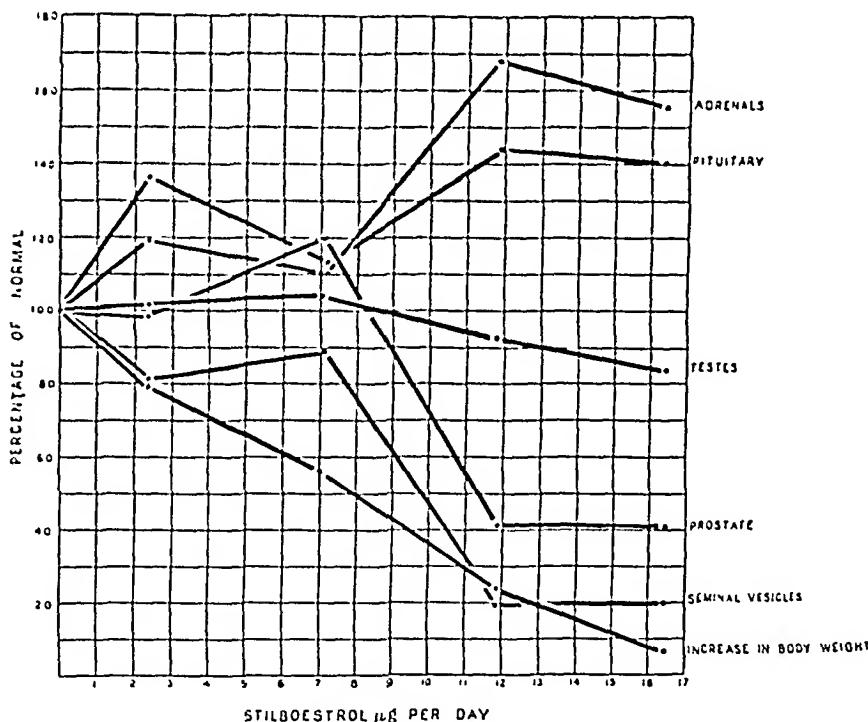
Fig. 1. Effects of oral administration of stilboestrol on growth and organ weights of adult male rats.

ment, were more uniform, although the individual variation of the animals was quite marked. It may be seen that a decrease in fluid intake occurred when the rats were transferred from the preliminary period on water to stilboestrol solution, and that this was most marked with the most concentrated solution used. The average weights of the various organs for the groups of rats on each solution, expressed in terms of 100 g. body-weight, have been calculated as the percentage of those obtained for the control animals. The average increase in body-weight over the 28-day period has been similarly expressed. These percentages were graphed against the daily average dose of stilboestrol, and are shown in Fig. 1.

It may be seen that the growth of the animal was affected by relatively small amounts of stilboestrol, and the effect produced appeared to be directly proportional to the daily dose of oestrogen. The weight of the

did not appreciably increase the effect on lactation. These results have been included in Table VI.

The rats in the litters which died during the experiment did not show any abnormality in the development of their reproductive organs. In female rats the uterus and vagina were not stimulated. The stilboestrol consumed by the parent rat apparently was not transmitted through the milk to the litter. When the young rats had reached the age of 18 days they were large enough to drink directly from the stilboestrol solution. In such cases uterine enlargement was noted in the female animals. As has been previously noted, growth of young rats is only slightly affected by oestrogen, and the amount consumed by them directly in these experiments would not appreciably affect their growth.

It was thought that rats in which lactation was partially suppressed by this method of stilboestrol administration might be a useful preparation on which to assay the effects of lactogenic hormone, especially since the stimulation produced by suckling would be a constant factor. Females with litters were therefore given a stilboestrol solution of 0·8 μg. per c.c. to drink, as above. The litters were weighed every other day, and when the average increase in weight became below 1·0 g. per day, the mother was injected with the preparation to be tested. When lactogenic hormone was injected into the mother in doses up to 30 units (Riddle's pigeon crop gland test) per day little effect was found in the growth of the litter. Adrenal cortical extract (Eucortone) given alone, or with prolactin, did not increase lactation. Finally, a suspension of adult rat pituitary glands was injected, at a dose equivalent to one gland a day, without producing increased lactation. If the stilboestrol solution were replaced by water it was found that lactation returned to normal, as indicated by the resumption of growth of the litters. These results have been recorded in Table VII.

Immature Rats

Since it has been shown that growth in the immature rat is only slightly decreased by oestrogen treatment [Noble, 1938 a, 1939] a number of immature animals were given a solution of 5 μg. per c.c. to drink. The growth of these rats was recorded over 10 weeks' time. The results obtained were essentially similar to those previously described following implantation of tablets of oestrogens into immature rats [Noble, 1939], and have not, therefore, been recorded in detail.

The young male rats grew at approximately half the normal rate up to about 140 g., and in the case of females, to about 110 g. After this, any increase in weight occurred very slowly. The gonads remained in an infantile condition due to the inhibition of gonadotrophic hormone of the pituitary gland.

testes, on the other hand, was little affected until the daily dose reached
7 μg. per day. Up to this dose an apparent increase occurred, because the
body-weight was reduced and the weights of the testes were expressed in
terms of body-weight. The weights of the prostate and seminal vesicles
were little affected up to 7 μg. per day, but as the dosage increased they
decreased rapidly, reflecting the failure of androgen secretion by the
testes. The weight of the pituitary and adrenals increased as the dose of
stilboestrol was raised.

Effects on lactation. In the preliminary experiments the rats, on the day
following parturition, were given stilboestrol solutions to drink of the
following concentration: 0·1 μg., 0·4 μg., 0·8 μg., 2·5 μg., and 5 μg. per c.c.
In each case the number of rats in the litter was limited to 5. The average
weight of the young rats and the number surviving at the end of each of
the 3 weeks after birth may be seen in Table VI. The average daily con-
sumption of stilboestrol by the mothers is also recorded.

Table VI. *Effect of oral administration of stilboestral to parent rats on the
growth of their litters. (The original number of rats in each litter was five)*

No. of litter	Parents Stilboestrol solution		Litters Average weight per rat.		
	μg. per c.c.	Av. daily dose per rat. μg.	7 days g.	14 days g.	21 days g.
4	0	0	14·4 [20]	23·7 [20]	38·7 [20]
4	0·1	2·7	11·6 [17]	15·0 [17]	26·0 [17]
4	0·4	9·6	9·9 [19]	14·2 [15]	18·4 [15]
10	0·8	17·5	10·9 [45]	12·6 [25]	14·8 [14]
3	2·5	62·2	10·3 [15]	11·6 [6]	12·3 [3]
3	5·0	64·3	8·1 [10]	—	—
*3	0·8	17·0	11·1 [15]	13·8 [14]	14·7 [13]
†2	5·0	49·5	5·6 [10]	6·0 [1]	—

[] = total number of rats in litters alive.
* Parent rats consumed an average of 11·5 μg. stilboestrol per day for 5 days prior to
parturition.
† Parent rats consumed an average of 30·0μg. stilboestrol per day for 5 days prior to
parturition.

These results show that when the adult animal was drinking as little as
2·72 μg. of stilboestrol a day the growth of her litter was below normal.
When larger amounts were consumed growth of the litter was correspond-
ingly retarded. When the mother was receiving 17·57 μg. per day the
survival of the litter was doubtful. The largest dose tested, however, did
not entirely suppress lactation. Six animals were started on stilboestrol
solution, 5 days prior to the birth of their litters. Although they con-
sumed from 11·5 to 30 μg. per day of stilboestrol over the 5 days, lactation
was not inhibited. Apparently the earlier start of the oestrogen treatment

did not appreciably increase the effect on lactation. These results have been included in Table VI.

The rats in the litters which died during the experiment did not show any abnormality in the development of their reproductive organs. In female rats the uterus and vagina were not stimulated. The stilboestrol consumed by the parent rat apparently was not transmitted through the milk to the litter. When the young rats had reached the age of 18 days they were large enough to drink directly from the stilboestrol solution. In such cases uterine enlargement was noted in the female animals. As has been previously noted, growth of young rats is only slightly affected by oestrogen, and the amount consumed by them directly in these experiments would not appreciably affect their growth.

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The young male rats grew at approximately half the normal rate up to about 140 g., and in the case of females, to about 110 g. After this, any increase in weight occurred very slowly. The gonads remained in an infantile condition due to the inhibition of gonadotrophic hormone of the pituitary gland.

testes, on the other hand, was little affected until the daily dose reached 7 μg. per day. Up to this dose an apparent increase occurred, because the body-weight was reduced and the weights of the testes were expressed in terms of body-weight. The weights of the prostate and seminal vesicles were little affected up to 7 μg. per day, but as the dosage increased they decreased rapidly, reflecting the failure of androgen secretion by the testes. The weight of the pituitary and adrenals increased as the dose of stilboestrol was raised.

Effects on lactation. In the preliminary experiments the rats, on the day following parturition, were given stilboestrol solutions to drink of the following concentration: 0·1 μg., 0·4 μg., 0·8 μg., 2·5 μg., and 5 μg. per c.c. In each case the number of rats in the litter was limited to 5. The average weight of the young rats and the number surviving at the end of each of the 3 weeks after birth may be seen in Table VI. The average daily consumption of stilboestrol by the mothers is also recorded.

Table VI. *Effect of oral administration of stilboestral to parent rats on the growth of their litters. (The original number of rats in each litter was five)*

No. of litter	Parents Stilboestrol solution		Litters Average weight per rat.		
	μg. per c.c.	Av. daily dose per rat. μg.	7 days g.	14 days g.	21 days g.
4	0	0	14·4 [20]	23·7 [20]	38·7 [20]
4	0·1	2·7	11·6 [17]	15·0 [17]	26·0 [17]
4	0·4	9·6	9·9 [19]	14·2 [15]	18·4 [15]
10	0·8	17·5	10·9 [45]	12·6 [25]	14·8 [14]
3	2·5	62·2	10·3 [15]	11·6 [6]	12·3 [3]
3	5·0	64·3	8·1 [10]	—	—
*3	0·8	17·0	11·1 [15]	13·8 [14]	14·7 [13]
†2	5·0	49·5	5·6 [10]	6·0 [1]	—

[] = total number of rats in litters alive.

* Parent rats consumed an average of 11·5 μg. stilboestrol per day for 5 days prior to parturition.

† Parent rats consumed an average of 30·0μg. stilboestrol per day for 5 days prior to parturition.

These results show that when the adult animal was drinking as little as 2·72 μg. of stilboestrol a day the growth of her litter was below normal. When larger amounts were consumed growth of the litter was correspondingly retarded. When the mother was receiving 17·57 μg. per day the survival of the litter was doubtful. The largest dose tested, however, did not entirely suppress lactation. Six animals were started on stilboestrol solution, 5 days prior to the birth of their litters. Although they consumed from 11·5 to 30 μg. per day of stilboestrol over the 5 days, lactation was not inhibited. Apparently the earlier start of the oestrogen treatment

but the total amount consumed was not appreciably increased. To establish this point, however, a careful fluid balance experiment was run on two pairs of male·rats. Each of these received 10 mg. of stilboestrol crystals implanted into the subcutaneous tissues. Such a procedure does not interfere with the health of the animal, and as has been shown [Noble, 1939], this substance rapidly disappears and produces only a temporary effect when administered by this method. The fluid balance and growth curve

Fig. 2. Growth and fluid exchange of two male adult rats after implantation of 10 mg. of stilboestrol crystals.

for one pair of these animals are represented in Fig. 2. Similar results were obtained for the other pair of rats.

It may be seen that an immediate lowering of the water intake and a fall in body-weight followed crystal implantation. As the effect of the stilboestrol diminished, as indicated by the increase in body-weight, the fluid intake also correspondingly increased. It would appear likely, therefore, that the decreased consumption of stilboestrol solutions noted in the rats was not necessarily related to the oral method of administration. If, however, the total fluid intake reaches a low level, a secondary effect on body-weight may occur due to lack of adequate fluid.

Hypophysectomized rats. To determine whether the effect of stilboestrol on fluid intake was related to its action on the pituitary gland, experiments were conducted on hypophysectomized rats. Following this operation in the rat a great increase in fluid intake and urine excretion occurs [Richter, 1934; Dodds, Noble, and Williams, 1937]. The polyuria and polydypsia

The immature animals appeared to differ from the adults in that there was very little alteration in fluid intake when the stilboestrol solution was substituted for water. It was always found that they consumed a normal amount of solution, even though it contained 5 μg. of oestrogen per c.c. It was possible, therefore, to maintain these animals on a daily dose of from 50 to 60 μg. of stilboestrol for prolonged periods.

Table VII. *Effect of treatment of parent rat (when lactation is reduced by the oral administration of stilboestrol) on the growth of their litter. (The average growth of control litters at a corresponding age was 1·6 g. per rat per day)*

	Parent rat			Litter Average weight change per rat	
Rat No.	Treatment with	Daily dose	Days of Treatment	During 4 days control period	During treatment of parent
				g.	g.
661	Prolactin	15 u.	6	−0·2	−1·0
671	,,	,,	8	+3·2	+1·0
674	,,	,,	8	+2·8	+2·0
663	,,	30 u.	8	+2·5	+5·2
673	,,	,,	8	−1·0	+3·4
662	Prolactin and 'Eucortone'	15 u. 0·5 c.c.	6	+0·4	−0·9
665	'Eucortone'	0·5 c.c.	8	+3·4	+2·0
675	Rat pituitary suspension	= 1 gland	8	+1·4	−3·0
677	,,	= ,,	8	−0·2	−0·9

Effect on Fluid Exchange

It may be seen from the results so far presented that the amount of fluid consumed rapidly fell off when stilboestrol was substituted for water. This decreased fluid intake appeared more marked as the concentration of the solution was increased. If this effect was primarily due to the rats disliking the solution for drinking purposes (a solution of 5 μg. per c.c. is tasteless to the human), the lowered fluid intake might primarily cause a reduction of body-weight. That a lowered fluid consumption may affect the animal has been found in control experiments. Male rats were allowed either 10 or 15 c.c. of water each per day for 28 days. In these cases body-weights were reduced below normal and a slight atrophy was found in the gonads of the animals receiving 10 c.c. per day. The reduced fluid intake after stilboestrol might, however, be secondary to the general effects of the oestrogen on the animal, possibly from a direct action on the metabolism or from the lowered pituitary function. This appeared to be the most likely explanation, since it was found that when rats which were drinking only small amounts of the more concentrated stilboestrol solutions were given access to water simultaneously, they drank both solutions,

out the total amount consumed was not appreciably increased. To establish this point, however, a careful fluid balance experiment was run on two pairs of male·rats. Each of these received 10 mg. of stilboestrol crystals implanted into the subcutaneous tissues. Such a procedure does not interfere with the health of the animal, and as has been shown [Noble, 1939], this substance rapidly disappears and produces only a temporary effect when administered by this method. The fluid balance and growth curve

Fig. 2. Growth and fluid exchange of two male adult rats after implantation of 10 mg. of stilboestrol crystals.

for one pair of these animals are represented in Fig. 2. Similar results were obtained for the other pair of rats.

It may be seen that an immediate lowering of the water intake and a fall in body-weight followed crystal implantation. As the effect of the stilboestrol diminished, as indicated by the increase in body-weight, the fluid intake also correspondingly increased. It would appear likely, therefore, that the decreased consumption of stilboestrol solutions noted in th rats was not necessarily related to the oral method of administration. I however, the total fluid intake reaches a low level, a secondary effect o body-weight may occur due to lack of adequate fluid.

Hypophysectomized rats. To determine whether the effect of stilboestr on fluid intake was related to its action on the pituitary gland, experiment were conducted on hypophysectomized rats. Following this operation i the rat a great increase in fluid intake and urine excretion occurs [Richte 1934; Dodds, Noble, and Williams, 1937]. The polyuria and polydypsi

appear within 4 hours after hypophysectomy, reach a maximum in 1 or 2
days, and then gradually decrease. Following hypophysectomy, there-
fore, four rats were given a solution of 5 μg. per c.c. of stilboestrol to drink.
The results were compared with those obtained for two control animals
run simultaneously, but which drank water, and are shown in Fig. 3.
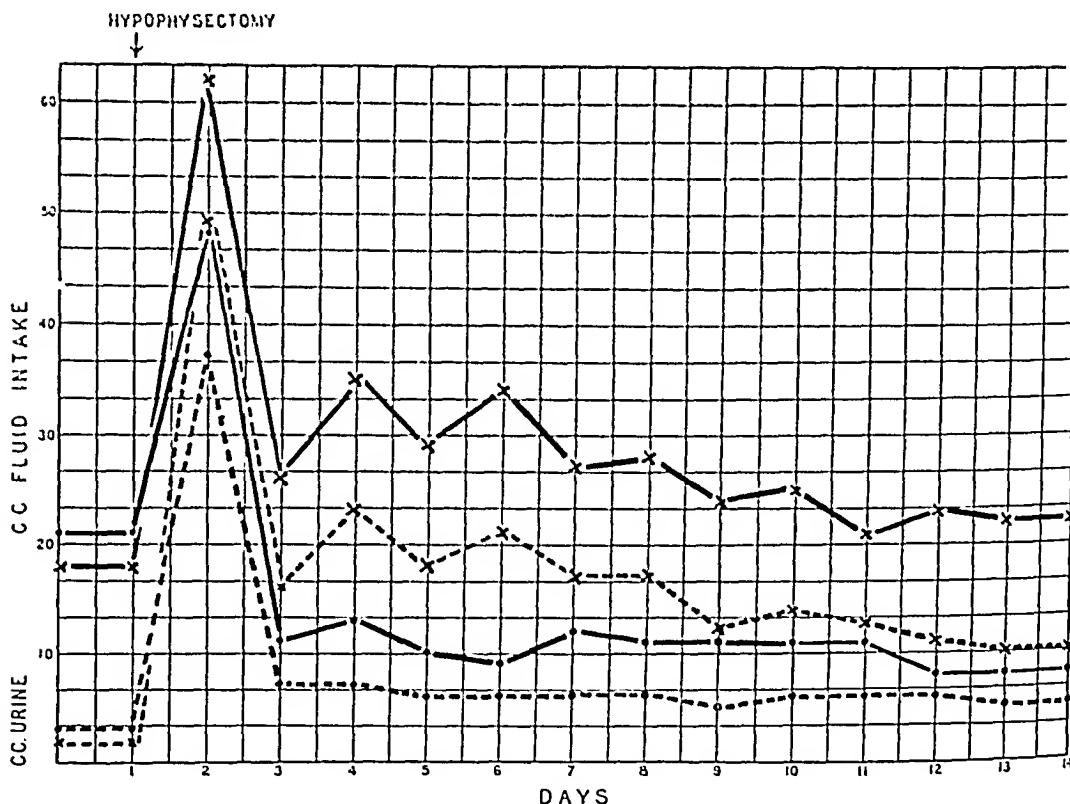
FIG. 3. Effect of oral stilboestrol administration on fluid exchange of rats following hypophysectomy.

X———X = Control water.
X----X = Control urine output.
●———● = Stilboestrol intake (5 μg. per c.c.).
●----● = Urine output.

In all the animals removal of the pituitary gland was followed by severe
polyuria and polydypsia. The fluid intake level of the four animals
receiving stilboestrol solution, however, returned to below normal on the
following day. The control animals, on the other hand, continued to show
a raised fluid intake level in 2 weeks' time. It would appear, therefore,
that stilboestrol may produce, even in the absence of the pituitary gland,
some alteration in metabolism, so that the animal requires a lowered fluid
intake. At autopsy, after 2 weeks, the rats receiving stilboestrol had a
definitely heavier average uterine weight (295 mg.) than the two controls
(125 mg.). No differences were found in the weight of the ovaries (treated

}·3 mg.—controls 23·5 mg.) or adrenal glands (treated 17·5 mg.—
)ntrols 16·0 mg.).

DISCUSSION

It has been shown that the administration of aqueous solutions of stil-
oestrol by the oral route to rats was followed by changes which were
ypical of those produced by oestrogen treatment. This method, while
eing relatively simple, has the added advantage that by varying the
oncentration of the stilboestrol solution the dose for the animal may
)e readily controlled over prolonged periods of time. From the results
)btained for male rats it has been shown that as small a dose as 2 to 3 μg.
)f stilboestrol daily may reduce the growth of the animal. As the dose was
ncreased the effect became more marked. The condition of the gonads
ippeared to remain normal until the animals received daily doses of over
7 μg. per day. The adrenals and pituitary gland showed an increased
weight when larger doses were employed. In lactating rats as low a dose
as 2 to 3 μg. per day appreciably reduced the growth of their litters. When
17 μg. or more per day was consumed the mortality in the litters was
greatly increased. It would appear, therefore, that of the various changes
produced by stilboestrol the effects on growth require the smallest dosage.
Lactation was reduced by doses of the same order as those which cause
decreased growth. Inhibition of the gonadotrophic hormones of the
anterior pituitary gland, with resulting atrophy of the gonads, was
observed when larger amounts of stilboestrol were given. The more or less
continuous absorption of oestrogen resulting from this form of administra-
tion is probably responsible for the small amounts of stilboestrol required
per day to exert the actions described. The oral dose of stilboestrol which
was necessary to produce vaginal oestrous in ovariectomized rats was
approximately 1 μg. [Dodds *et al.*, 1938]. These results, therefore, would
suggest that oestrogens under normal conditions may be present in suffi-
cient amount in the animal to exert an influence on body-growth and the
secretory activity of the pituitary gland.

Whereas the action of oestrogens on the gonads seems to be indirectly
produced through the inhibition of gonadotrophic hormone secretion by
the pituitary gland, body-growth and lactation may be directly affected.
The observation that once lactation was reduced by oestrogens it could not
be increased by anterior pituitary extracts is suggestive that the primary
effect of the oestrogen was on the mammary tissue and not on the pituitary
gland. It is possible, however, that the amount of extract given was not
sufficient to stimulate lactation. That the mammary glands were in a con-
dition so that they could yield more milk was shown by their increased
secretion when oestrogen treatment was discontinued. It was not found

possible to inhibit lactation completely, even when stilboestrol was administered to the female rat before parturition. The inhibition of lactation in the rat by injections of stilboestrol has been previously studied by Folley and Watson [1938].

Further evidence that oestrogens may exert a direct action on the tissues of the rat which is independent of the pituitary gland has been given. The fall in body-weight which follows stilboestrol treatment in adult rats was found to be associated with a parallel decrease in fluid intake. A similar finding was noted when stilboestrol was administered as crystals implanted into the subcutaneous tissues. The behaviour of the immature rat was quite different from that of the adult. Body-growth was only slightly retarded by stilboestrol treatment and a reduction in fluid intake did not occur. Oestrogens may exert an influence on fluid exchange in the rat even after removal of the pituitary gland. In the experiments described stilboestrol prevented the prolonged polyuria and polydypsia which follow hypophysectomy in the rat. These findings were of interest in view of the investigations by Zuckerman, Palmer, and Bourne [1939], who showed that the water-content of the skin, uterus, and vagina in rats was increased following the injection of oestradiol.

SUMMARY

By substituting aqueous solutions of different concentrations of stilboestrol for rats' drinking water it has been possible to administer this substance at various dose levels for prolonged periods of time. In adult male animals a daily dose of 2 to 3 μg. of stilboestrol caused a slight but definite inhibition of body-growth, whereas atrophy of the gonads was not produced until a daily dose of approximately 7 μg. was given. Lactation was reduced by a daily dose of 2 to 3 μg. Larger doses of stilboestrol were followed by enlargement of the adrenals and pituitary gland, marked atrophy of the gonads, and extreme alterations in body growth and lactation.

A reduction in fluid intake occurred in adult rats after the oral administration of aqueous solutions of stilboestrol, or following implantation of crystals. Such an effect was also observed in rats after hypophysectomy. Immature rats did not exhibit a lowered fluid intake even though they consumed a solution of stilboestrol containing 5 μg. per c.c. Body-growth of young rats was only partially retarded.

Lactation when reduced by oral stilboestrol administration was not restored following treatment with anterior pituitary extracts, but returned to normal when the stilboestrol was discontinued.

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THE DURATION OF ACTION OF CERTAIN NATURAL AND SYNTHETIC OESTROGENS WHEN ADMINISTERED ORALLY OR BY INJECTION

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It has been shown by Dodds, Golberg, Lawson, and Robinson [1938] that certain di-esters of diethylstilboestrol have a prolonged action when injected into spayed rats, as have the esters and di-esters of naturally occurring oestrogens. Maximum prolongation of oestrus occurred, however, with the dipropionate, not with the higher esters (dibutyrate and divalerate) of the fatty acid series. This is in contrast to the behaviour of the esters of oestrone and oestradiol, of which the higher esters have a more prolonged action than the lower ones [Miescher, Scholz, and Tschopp, 1938 *a*, *b*, *c*]. The dibenzoate and dimethyl ether of diethylstilboestrol also had prolonged oestrogenic activity when given in doses of 100 μg. or more. Robson, Schönberg, and Fahim [1938], using criteria other than full cornification, have reported that large doses of oestradiol benzoate-butyrate (500 μg.), of triphenyl ethylene (10,000 μg.), and of triphenyl chlorethylene (500 μg.), injected into spayed mice had an oestrogenic action lasting for several months, while 500 μg. of free diethylstilboestrol or oestradiol benzoate had a total duration of effect of 27 and 38 days respectively. When given orally in the same dosage, they found that these compounds had an effect lasting between 13 and 25 days. Kreitmair and Sieckmann [1939] compared the activities of diethylstilboestrol and its diacetate and dipropionate, oestrone and oestradiol benzoate, when given by injection to spayed rats and mice, and when given orally to rats. The durations of action of diethylstilboestrol and oestrone by injection were found to increase but little with increasing dosage, the oestrogenic effect of 13 μg. (100 times the dose required to give 50% of positive reactions) lasting for 3–4 days only. The diethylstilboestrol di-esters and oestradiol benzoate, on the other hand, showed prolonged activity with the higher doses. None of the substances had a markedly prolonged action when 100 'oral rat units' were given by mouth, though diethylstilboestrol dipropionate gave an effect lasting for 3–6 days.

TECHNIQUE

In the present experiments, a further comparison of the duration of action of some of these, and of other oestrogens, has been made. The

substances have been given by two injections or two doses by mouth to spayed mice, one dose on each of two consecutive days, as described elsewhere [Emmens, 1939 b]. Smears have been taken twice daily on the 3rd and 4th day after the first injection, and once daily thereafter until four or more consecutive negative smears occurred. The criterion of oestrus adopted has been the presence of cornified epithelial cells in the absence of leucocytes. All solutions were made in nut oil, the injection or feeding volume being between 0·025 and 0·2 ml.; each dose was tested on from 1 to 5 mice. For the purposes of this investigation it was considered sufficient to estimate the relative potencies of the compounds in a very approximate manner. They have usually been administered by series of graded doses in the ratios 1 : 2 : 4, &c., the lowest effective dose in each series being taken as a basis for a comparison of activity. These doses are given in Tables I and II, for subcutaneous injection and peroral administration respectively.

Table I. *The duration of action, in days, of various oestrogens when given by subcutaneous injection to ovariectomized mice. Doses are shown in terms of the lowest effective dose of each preparation*

Substance	Lowest effective dose in μg.	Dose (as multiple of lowest effective dose)									
		1	2·5	5	10	20	40	100	200	2000	8000
Diethylstilboestrol	0·1	2	1	2	—	—	—	5	7	—	14
Diethylstilboestrol dipropionate	0·25	6	5–6	6	6	9	17				
Diethylstilboestrol dibenzoate	20·0	21	29	34	30	28					
Diethylstilboestrol dipalmitate	50·0	18	30	38							
Diethylstilboestrol dimethyl ether	5·0	1	6	11	12						
Oestrone	0·1	½	—	—	—	4	4	—	—	7–8	
Oestrone methyl ether	1·25	1	1	4	6	6	14				
Ethinyl dihydroequilin	0·05	½	½–1	1	2	2	2	3			
Ethinyl oestradiol di-n-butyrate	2·0	10									

RESULTS

A comparison of the doses of diethylstilboestrol and its esters, required by mouth and injection respectively, brings out the interesting point that, while very considerable differences exist between the lowest effective doses of the different substances by injection, such differences are not seen when the compounds are given orally. Furthermore, the higher esters of diethylstilboestrol and also ethinyl oestradiol di-n-butyrate are actually more active by mouth than by injection, diethylstilboestrol dipalmitate 50 times so. These facts illustrate very clearly the mode of action of esterification in producing a prolonged action, shown by Deanesly

and Parkes [1937] to be due to delay of absorption from the site of injection. With the methyl ethers, which do not show great differences between their oral and parenteral activities, a different state of affairs is found. Diethylstilboestrol dimethyl ether must be given orally in a significantly higher dose than diethylstilboestrol, but the lowest effective dose of oestrone methyl ether is approximately the same as that of oestrone. By injection, on the other hand, in both cases, more than 10 times the dose is necessary with the methyl ethers than with the parent substances.

Table. II. *The duration of action, in days, of various oestrogens when given by mouth to ovariectomized mice. Doses are given in terms of the lowest effective dose of each preparation*

Substance	Lowest effective dose in µg.	Dose (as multiple of lowest effective dose)							
		1	2·5	5	10	20	40	200	3200
Diethylstilboestrol	0·5	½	—	—	—	2	3	—	7–8
Diethylstilboestrol dipropionate	0·5	½	1	2	2	2	3		
Diethylstilboestrol dibenzoate	1·0	½	2	1	2	3			
Diethylstilboestrol dipalmitate	1·0	½	¼	1–2	3	3	2		
Diethylstilboestrol dimethyl ether	2·5	2	2	1–2	4	4	3–4		
Oestrone	2·0	½	—	—	—	—	—	5	
Oestrone methyl ether	2·5	½	1–2	1	—	2	2		
Ethinyl dihydroequilin	2·5	½	1	1					
Ethinyl oestradiol di-n-butyrate	0·5	½	1	1					

The duration of action, in days, of the higher doses of each compound is given in Tables I and II, in which the doses are expressed in terms of the lowest effective dose of each substance. Given by injection, the esters show the expected prolonged action, and increasing the dose is relatively less effective as the threshold dose becomes higher. In the case of diethylstilboestrol dibenzoate and dipalmitate, it was difficult to estimate the total duration of action owing to the occurrence of the phenomenon already described by Castillo and Calatroni [1930] and Zuckerman [1938]. These authors found that cornification of the vaginal contents recurs periodically in spayed rats injected with a constant low daily dose of oestrone. Following the two doses of the esters, there was an irregular cyclic appearance of leucocytes, sometimes with an almost complete disappearance of cornified cells, after which full vaginal cornification was resumed. The mice receiving these substances were therefore examined for 8 to 10 days after the presumed cessation of reactions, in order to be sure that cornified smears did not recur. The explanation of this happening seems to be that, with medium doses of the very slowly absorbed diesters, an almost minimal degree of stimulation may be maintained over a considerable period. Crystals of oestrone, weighing 0·25–8 mg., have, when implanted subcutaneously into spayed mice, given rise to the same

phenomenon. Cornification is complete for a period of several weeks, and is followed by a long period during which periodic invasions of leucocytes are seen. The cessation of cornification was usually quite sharp in the animals receiving the other compounds, and occurred very regularly in mice on the same dose of any given substance. Esterification of the oestrogens, therefore, raises the threshold dose by injection, but not necessarily by mouth, and prolongs their action by injection, but not (see below) by mouth. The prolonged action shown on injection of the higher doses of the methyl ethers of diethylstilboestrol and of oestrone is presumably due to the slowness with which the body effects hydrolysis of such ethers, without which they cannot be esterified and excreted.

Wade and Doisy [1935] showed that excessive smearing may cause vaginal cornification in uninjected rats. Although smears were not taken with undue frequency, it seemed possible that daily smearing might increase the apparent duration of action of those substances with which oestrus was prolonged. In the case of mice receiving the highest doses of diethylstilboestrol and oestrone by injection, the 5 mice on each dose were therefore divided into two groups of 2 and 3 mice each, the first of which, group A, was smeared daily as usual, the second, group B, was left without smears being taken for a week after the first appearance of cornification. With oestrone, group A was still positive in reaction on the 7th day, but group B gave negative smears, which, however, contained many cornified cells and only a moderate number of leucocytes, and had apparently only just become negative. On the 8th day, all mice gave negative smears. With diethylstilboestrol, both groups gave positive smears on the 7th day after the beginning of cornification. Group B was again left without smears being taken, until both mice in group A simultaneously gave a negative smear, which occurred on the 15th day, whereupon group B was examined and found to give negative smears. These smears also contained many cornified cells and few leucocytes, and had clearly only just become negative. There seems, therefore, to have been a slight, but not serious, prolongation of effect due to the taking of smears.

The three non-esterified oestrogens, diethylstilboestrol, oestrone, and ethinyl dihydroequilin, despite their different chemical constitutions are seen to be similar in their duration of action when injected into spayed mice. Up to 40 times the lowest effective dose exerts an action over 4 days or less, and 200 or more times this dose is needed to give an action lasting for a week. Diethylstilboestrol has perhaps a slightly more prolonged effect than the others. When given orally, both the esterified and non-esterified compounds have a duration of action a little shorter than that of the injected non-esterified compounds, when corresponding doses in terms of the minimum effective dose are compared. Diethylstilboestrol

dimethyl ether has a slightly more prolonged action than the rest, but it seems that the solution of the problem of finding an oestrogen which is active for a long period when given by mouth is not yet obvious and does not lie in the use of esterified compounds. As with ethinyl oestradiol [Emmens, 1939 a], ethinyl dihydroequilin does not show an oral activity in mice which is comparable with its activity by injection. The ratio of the oral to parenteral dose for both of these compounds is similar to that for free oestradiol, being between 45 and 60. This seems to be due to a species difference, as ethinyl oestradiol, when tested on spayed rats by Inhoffen, Logemann, Hohlweg, and Serini [1938], was found to be 1/30th, and ethinyl dihydroequilin 1/20th as active by mouth as by injection, while oestradiol is only 1/500th as active, all three substances having the same potency by injection (one 'rat unit' = 0·1 μg.).

SUMMARY

A comparison of the duration of action of a number of natural and synthetic oestrogens shows that, while esterification is effective in prolonging the duration of action by injection, it does not do so when the compounds are given orally. Moreover, the minimum effective dose of an orally administered ester is similar to that of the non-esterified parent substance.

Diethylstilboestrol dimethyl ether given by mouth shows a slight prolongation of effect when compared with diethylstilboestrol in terms of the minimum effective doses.

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THE EFFECT OF SEX HORMONES, CORTIN, AND VASOPRESSIN ON WATER-RETENTION IN THE REPRODUCTIVE ORGANS OF MONKEYS

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IT has recently been found that sex hormones, like the hormone of the adrenal cortex, have water- and salt-retaining properties. Thus retention of water can be demonstrated both in normal monkeys during the follicular phase of the menstrual cycle [Krohn and Zuckerman, 1937] and in spayed monkeys during periods of experimental oestrogenic stimulation [Guthkelch and Zuckerman, 1937]. In male and female dogs it occurs not only as a result of oestrogenic stimulation but also in consequence of injections of progesterone, testosterone, or pregnandiol [Thorn and Harrop, 1937; Thorn and Engel, 1938; Thorn, Nelson, and Thorn, 1938]. Unlike the rat, in which retention of water affects most organs and tissues [Zuckerman, Palmer, and Bourne, 1939], the water which is retained by some species of monkeys as a result of oestrogenic stimulation appears to be mainly deposited in the sexual skin, and this fact made it possible to devise the following experiments to differentiate between the water-retaining properties of cortin, oestrone, and testosterone. The first object of these experiments, which have been referred to in a preliminary abstract [Zuckerman, 1938], was to discover whether water that had accumulated in the sexual skin as a result of oestrogenic stimulation remained there when cortin or testosterone was given daily immediately after the end of the oestrone treatment. Further experiments were carried out with vasopressin, which also diminishes the amount of water excreted by the body. None was carried out with progesterone in view of the fact that the normal sexual-skin cycle in chimpanzees, baboons, and such species of macaque as the pig-tailed monkey, shows that the progesterone produced by the corpus luteum is unable to retain, in the sexual skin, water which has been deposited there as a result of oestrogenic stimulation in the pre-ovulatory (follicular) phase of the cycle. What water-retaining properties progesterone may possess clearly do not normally apply to the sexual skin.

These experiments threw light on a second problem. The endometrium in monkeys and human beings becomes oedematous during the proliferative and premenstrual phases of the cycle, as it also does in animals such

as rabbits when injected with oestrogenic hormone [Fagin and Reynolds, 1936], and it has been shown experimentally that oestrogenic stimulation leads to the retention of water in the uterus [Astwood, 1938; Zuckerman, *et al.*, 1939; Astwood, 1939, working on the rat]. Water-retention and oedema are thus in some way concerned in the building-up of the endometrium. Since a sub-threshold fall in the level of oestrogenic stimulation leads to uterine bleeding, and since the onset of bleeding in monkeys is associated with dehydration of the uterus [Van Dyke and Ch'en, 1936], it would seem that the actual breakdown of the endometrium may be due, at least partly, to resorption of water which, having accumulated in the stroma under the influence of oestrogenic and, in cycles with ovulation, possibly progestational stimulation, acted as a fluid scaffolding of the proliferated glands and dilated vessels. Such a view is also suggested by histological observations made by Markee [1938], who writes that withdrawal of oestrogen or progesterone causes regression and thinning of the endometrium, with a resulting disproportion between the thickness of the endometrium and the length of the coiled arteries, whose consequent compression leads to vascular stasis and degeneration. It was therefore of interest to inquire whether or not 'post-oestrogen' bleeding could be inhibited if a series of oestrone injections was immediately followed by daily treatment with some substance, other than oestrone, with salt and water-retaining properties (e.g. cortin, testosterone).

EXPERIMENTAL

Four fully mature spayed pig-tailed monkeys (*Macaca nemestrina*, a species of macaque which experiences pronounced sexual-skin swelling) and six mature and adolescent spayed rhesus monkeys (*Macaca mulatta*) were used in this study. Observations were also made on a normal pigtailed macaque and two immature Guinea baboons (*Papio papio*). Except where otherwise stated, the animals were injected once daily. The sex hormones were given intramuscularly in oil solution, while cortin and vasopressin were injected subcutaneously. The vasopressin used was the Allen and Hanbury preparation assayed at 20 units per c.c. One preparation of cortin was obtained from Allen and Hanbury ('Eucortone'), 1 c.c. being equivalent to 75 g. of adrenal cortex. A second preparation of cortin was obtained from the Upjohn Company, U.S.A. Each c.c. was equivalent to 40 g. of fresh beef adrenal gland, and was assayed to contain four (survival) rat units per c.c. [Cartland and Kuizenga, 1936]. A series of tests showed that 0·5 c.c. of this preparation is sufficient to maintain oestrous cycles in adrenalectomized mature female rats.

RESULTS
Sexual Skin
Oestrone.

Exp. 354. Oestrone was given daily in increasing doses (100 μg.–400μg.) to a normal mature pig-tailed monkey that weighed 7 kg. Injections were begun on the day after the beginning of subsidence of the sexual skin, i.e. approximately 2 days after ovulation and at the time of maximum excretion of urine in the cycle. The sexual skin gradually expanded, and was fully swollen 14 days after the beginning of injections.

This experiment, like that on a sooty mangabey (*Cercocebus torquatus atys*) reported previously [Zuckerman, 1935], shows that oestrone given in the second half of the menstrual cycle can reverse the normal order of events and lead to the redeposition of water in the sexual skin.

Two further experiments (244 and 245), carried out on immature Guinea baboons (*Papio papio*), show that the sexual skin, in species in which the mature female normally shows cyclical sexual-skin swelling, will remain swollen as long as oestrogenic stimulation lasts. In the one experiment the injections were continued for 233 days, and in the other for 279 days. It may be noted that at no time did the swelling exceed that observed in pubertal baboons of this species.

Testosterone Propionate

Exp. 366. Oestrone was injected for 39 days in increasing doses (100 μg–300 μg.) into a mature spayed pig-tailed monkey that weighed 5 kg. The sexual skin was slow in responding, but at the conclusion of this phase of the experiment it was almost as swollen as it ever becomes in this species of monkey [Krohn and Zuckerman, 1937]. The animal was given 100 mg. of testosterone propionate on the 40th, 43rd, and 46th days of the experiment. By the 43rd day the swelling of the sexual skin had decreased considerably, and by the 47th day the skin had assumed its fully quiescent appearance.

The amount of testosterone propionate which this animal received (300 mg. in one week) is far above the level (15 mg. per week) necessary to inhibit the menstrual cycle in a mature rhesus macaque [Zuckerman, 1937 a; also further unpublished observations]. Consequently it may be concluded that testosterone, like progesterone, is incapable of retaining, in the sexual skin, water that has been deposited there as a result of the action of small doses of oestrone (1 to 2 mg. of oestrone per week). On the other hand, it may be observed that it is capable of causing reddening of the sexual skin in the female rhesus macaque [Zuckerman, 1937 d; Hartman, 1937].

L

Cortin

Exp. 370.2. 100 μg. of oestrone were injected daily for 28 days into a mature pig-tailed macaque that weighed 4·7 kg. At the end of this phase of the experiment the swelling of the sexual skin was maximal. From the 29th day until the 36th day the animal was given 10 c.c. of the Upjohn preparation of cortin daily (equivalent to a total of 3·2 kg. fresh beef adrenal gland). Subsidence of the sexual-skin swelling began on the 3rd day of cortin injections and was complete by the 7th day. Uterine bleeding set in on the 8th day (the 36th day of the total experimental period). The mean daily excretion of urine during the period of cortin treatment was 75·7% higher than it was during the last 10 days that oestrone was administered.

Exp. 357. A similar result was obtained in a second experiment on a mature pig-tailed macaque that weighed 5·1 kg. Subsidence of the sexual skin began on the 2nd day of cortin injections, and the mean daily excretion of urine during the period of cortin injections was 71·9% higher than it was during the last 10 days of oestrone injections.

Extracts of cortin vary considerably in their potency, and at present the strength of commercial extracts of cortin is not equated with that of crystalline corticosterone or desoxy-corticosterone. The doses given in the present experiments were large, however, not only from the experimental and clinical point of view but also in relation to the body-weight of the animals. The conclusion may therefore be drawn that in doses which are able to cause considerable salt- and water-retention in dogs and in human beings, cortin cannot hold back water that has been deposited in the sexual skin as a result of oestrogenic stimulation.

Vasopressin

The administration of vasopressin to normal animals is rapidly followed by considerable diminution in the amount of urine excreted; this effect is generally attributed to direct action upon the kidneys. It was consequently of interest to inquire how the sexual skin would respond when vasopressin is administered to a pig-tailed monkey immediately after the cessation of oestrone treatment.

Exp. 370.3. A spayed pig-tailed macaque weighing 5 kg. was injected with 100 μg. of oestrone daily for 20 days. At the end of this phase of the experiment the sexual skin of this animal had swollen to its maximal proportions. Beginning on the 21st day 2 units of vasopressin were injected every 4 hours throughout the day and night. 0·25 unit of vasopressin injected into a man will delay for 3 to 4 hours the diuresis which otherwise follows the drinking of water [Burn, 1928]. Relative to its body-weight, the monkey used in this experiment thus received very large doses

of the hormone (the tolerance of rhesus monkeys to injections of posterior pituitary extract has been commented on by Hartman and Geiling [1936], who injected as much as 464 units in a period of 5 hours).

No change was observed in the sexual skin of the pig-tailed monkey until the end of the 5th day of vasopressin injections, when the swelling began to subside. By the end of the 7th day the sexual skin had regressed to its normal resting condition. The injections were continued in the same way for a further 2 days, uterine bleeding beginning on the 9th day of vasopressin injections.

The volume of urine fell immediately the vasopressin injections were begun, the mean daily excretion during the 9 days of injection being 50% less than during the part of the experiment when oestrone was injected. A great increase in the amount of urine excreted occurred on the day before uterine bleeding began, but the volume fell again when oestrone injections were restarted during the phase of bleeding.

This experiment shows that the normal responses of the sexual skin altered as a result of the redistribution of body-fluids caused by the vasopressin anuria. Had the vasopressin not been administered, the sexual skin would have subsided and the water which it contained would have been excreted much sooner than it was. It also follows that in the absence of oestrogenic stimulation, the sexual skin cannot indefinitely continue to hold water in spite of the considerable increase in the water-content of the body which results from anuria. The increase in the amount of urine excreted on the 8th day of the vasopressin injections was clearly correlated with the subsidence of the sexual skin which began 3 days before.

The Uterus

Oestrone

Observations published elsewhere [Zuckerman, 1937 *b*], show that uterine bleeding will not occur in spayed monkeys which are injected daily with a sufficiently high dose of oestrone (experimental period up to one year). They also show that the bleeding which would normally occur after the cessation of oestrogenic stimulation can be inhibited if injections of oestrogenic hormone are restarted up to, and sometimes even after, the middle of the latent period following the course of injections.

Further observations made during the course of the present study show that the normal period of uterine bleeding after a phase of oestrogenic stimulation (12 observations: $6\cdot8\pm0\cdot34$ days) is significantly shortened if a large dose of oestrogenic hormone is administered on the 1st or 2nd day of bleeding (27 observations: $2\cdot81\pm0\cdot15$ days).

Progesterone

It is well established [Smith and Engle, 1932; Hisaw, 1935; Zuckerman, 1937 c] that the uterine bleeding which normally follows the cessation of a series of oestrone injections can be inhibited by the administration of progesterone. The period of inhibition apparently lasts for as long as sufficient progesterone is administered.

Testosterone Propionate

The following experiments, like two reported by Hartman [1937], show that testosterone propionate will also inhibit the uterine bleeding which normally follows the cessation of a course of oestrone injections.

Exp. 3.39. 100 μg. of oestrone were injected daily for 14 days into a fully mature spayed rhesus monkey that weighed 5·5 kg. Beginning on the 15th day of the experiment, 50 mg. of testosterone propionate were injected every 3rd day for 26 days. Uterine bleeding did not occur at any time during this phase of the experiment. It set in for the first time 12 days after the cessation of the testosterone treatment (the 50th day of the whole experiment).

A similar result was obtained in two other experiments of the same kind (68.28, 69.29).

It may be noted that testosterone propionate in the amounts given in these experiments does not lead to progestational differentiation of the endometrium [Zuckerman, 1937 a].

Cortin

The following experiments show that in the relatively high amounts administered, cortin is unable to inhibit the uterine bleeding which occurs after a course of oestrone injections.

Exp. 211.1. 50 μg. of oestrone were administered daily for 14 days to an almost mature spayed rhesus monkey that weighed 3·5 kg. From the 15th day 2 c.c. of the Upjohn preparation of cortin were injected daily. Uterine bleeding set in on the 9th day of the cortin injections (24th day of the experiment).

In three similar experiments (211.3, 275.1, 3.38) in which larger doses of cortin were given daily (up to 10 c.c. of the Upjohn preparation daily, equivalent to a total of 2·4 kg. of fresh beef adrenal gland) similar results were obtained, bleeding setting in on the 6th, 6th, and 8th days of cortin injections respectively. Uterine bleeding also occurred during the course of cortin injections in experiment 370.2 described above.

Vasopressin

Hartman and Geiling [1936] have shown that relatively large doses of posterior pituitary extract do not stimulate uterine bleeding. The follow-

ing experiments show that the injection of vasopressin after a phase of oestrogenic stimulation does not inhibit the occurrence of uterine bleeding.

Exp. 203.1. 100 μg. of oestrone were injected daily for 14 days into a fully mature spayed rhesus monkey that weighed 5·3 kg. Beginning on the 15th day, 1 unit of vasopressin was injected 4-hourly throughout the day and night for 4 days. Uterine bleeding began in the late afternoon of the 4th day (18th day of the experiment). Anuria occurred during the period of vasopressin treatment.

The same result was obtained in two other similar experiments (203.1, 370.3).

DISCUSSION

The water-retaining properties of the sex-hormones may in general be related to the chemical similarity of these substances to corticosterone and desoxy-corticosterone. The experiments reported in this paper make it clear, however, that there are considerable differences in the manner of action of these various substances. Thus while observations on the renal excretion of sodium by dogs suggest that progesterone is nearly twice as potent as oestrone in its general salt- and water-retaining properties [Thorn and Harrop, 1937], unlike oestrone, it has no water-retaining effect on the sexual skin. Unlike oestrone, too, progesterone, like cortin, has no appreciable effect on the renal excretion of inorganic phosphorus and total nitrogen [Thorn and Engel, 1938]. Moreover, while oestrone and testosterone propionate resemble each other in reducing the excretion of inorganic phosphorus and total nitrogen, they differ profoundly from each other in so far as testosterone propionate has no water-retaining effect on the sexual skin, which responds to its stimulation only by reddening. The intense water-retaining effect of oestrogenic stimulation on the skin must therefore be regarded as a specific response, and one that can perhaps be related to the peculiar cytological changes which occur in the sexual skin when it is under the influence of such stimulation (e.g., enlargement of the fibroblasts [Aykroyd and Zuckerman, 1938]). Whether or not the retention of nitrogen as a result of oestrogenic stimulation can be accounted for by these cytological changes remains to be determined.

If the resorption of water in the endometrium is regarded as one of the immediate causes of tissue breakdown at menstruation, it is plain, too, that this effect is specific, for, as the above experiments show, cortin appears to be ineffective in retaining in the endometrium water that has accumulated there as a result of oestrogenic, and probably luteal, stimulation. In this respect the sexual skin appears to differ from the uterus, for

if dehydration of the endometrium is a necessary condition of uterine bleeding, it would seem that progesterone and testosterone can stimulate the retention of water in the endometrium although they cannot do so in the sexual skin.

SUMMARY

1. Large doses of oestrone injected daily during the post-ovulation phase of the menstrual cycle of the pig-tailed monkey lead to swelling of the sexual skin. The swelling is due to the accumulation of water in the skin.

2. The fact that swelling does not occur during this phase of the normal cycle shows that the progesterone produced by the corpus luteum is unable to retain in the sexual skin the water deposited there as a result of oestrogenic stimulation during the pre-ovulation phase.

3. Testosterone propionate and cortin injected into spayed monkeys do not lead to the retention, in the sexual skin, of water that has accumulated there as a result of previous injections of oestrone.

4. The injection of vasopressin at short intervals delays the resorption of sexual-skin swelling.

5. Neither cortin nor vasopressin appears to be able to inhibit post-oestrogen bleeding in monkeys. Both progesterone and testosterone do.

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PRECIPITINS IN ANTIGONADOTROPHIC SERA

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COLLIP and Anderson [1934] first demonstrated the antihormone properties in the serum of rats receiving prolonged courses of injections of anterior pituitary extract. Since then such inhibitory sera to many different pituitary and other hormones have been prepared in a variety of animal species. Opinion is still divided as to the nature of the inhibitory substances. Collip and his co-workers regard them as antagonistic hormones, basing their views mainly on the results of experiments which show that an animal can produce inhibitory substances to pituitary hormones of its own species [Selye, Collip, and Thompson, 1934; Collip, 1937], and believe that such inhibitory substances may be normally present in the serum. Results obtained by other investigators, however, show that continued injection of hormone extracts from a species foreign to that used for the production of antihormones is essential [Katzman, Wade, and Doisy, 1937; Thompson, 1937].

There is much in favour of the view that the inhibitory substance is a true antibody. Thus the physico-chemical properties have been thoroughly investigated by Harington and Rowlands [1937], as well as Zondek and Sulman [1937 a, b] and Zondek, Sulman, and Hochman [1938 a], and found to correspond closely to those of antibodies in general. The importance of an active reticulo-endothelial system for the production of antihormones, as shown by Gordon, Kleinberg, and Charipper [1937], also favours an immunological mechanism.

In most experiments, crude pituitary extracts have been used for immunization. The antisera so obtained, in spite of a marked inhibitory effect on the hormonal activity of the crude extracts, may fail to reduce the response to more highly purified extracts from the same source. It has been stated, indeed, that such highly purified extracts do not evoke the formation of inhibitory substances when injected in the same dosage and over a similar period of time [Werner, 1936 a, b]. Brandt and Goldhammer [1938 b] found that a heat-inactivated hormone was as capable of producing the inhibitory substance as the unheated preparation. Zondek *et al.* [1938], on the other hand, hold that boiling does not completely inactivate the urinary gonadotrophin (prolan), and that the power of stimulating the production of antihormones depends on the residue of

active hormone left. Their work, however, does not provide conclusive evidence that hormone activity is essential for immunization. Toxoids which have lost the specific toxicity of the parent toxins act excellently as antigens for the production of antitoxins; and the fact that boiling does not necessarily destroy the antigenic power of a protein is shown by the ability of boiled proteins to cause specific sensitization of guinea-pigs [see Doerr, 1929]. It is possible, therefore, that the boiled prolan used in the experiments of Zondek *et al.* contained, in addition to the residue of active hormone, a proportion of inactivated material still capable of stimulating the production of antibodies to the hormone.

Even if Werner and others are right in their conclusion that hormone activity is not essential as a stimulus for antihormone production, this would not exclude the possibility that the inhibitory substance may be a true antibody.

The possibilities of the participation of a hormone in antigenic activity are, indeed, somewhat various and complicated. Thyroxine is a peculiar amino-acid entering into the structure of the thyroid protein, thyroglobulin. Stokinger and Heidelberger find [1937] that a thyroglobulin has antigenic specificities related to the species, and to the organ of its origin, and that these are due to its whole molecular pattern and are not specially determined by the iodine-containing amino-acids which enter into its constitution. Rosen and Marine [1937] found that rabbits could produce a precipitating antibody to ox thyroglobulin, but that its presence did not interfere with the rise in basal metabolic rate produced by an injection of the same thyroglobulin. Stokinger and Heidelberger found that thyroxine did not react with or interfere with the action of a precipitin for a thryoglobulin. In such natural combination, therefore, thyroxine appears to act merely as a constituent amino-acid of a complex protein antigen, and not as a hapten. Clutton, Harington, and Yuill [1938], on the other hand, have shown that thyroxine can act as a hapten when artificially combined with proteins, so that an antiserum prepared against such a complex artificial antigen will neutralize the physiological action of thyroxine itself, though producing no visible precipitate with it. This last observation may have great significance for our problem of the nature of the antihormones. Free hormones of the chemical simplicity of thyroxine, or oestrone, would not be expected to act as antigens. In the case of insulin, which is a relatively complex peptide, there are observations on record suggesting its action as an anaphylactic antigen, with an identical specificity whatever the species of its origin [Lewis, 1937]. These observations need confirmation, since it is as difficult with insulin as with hormones of simpler constitution to imagine that an animal will produce an antibody in response to a single injection of a substance which

the endocrine system is constantly injecting into its circulation. Any such hormone, on the other hand, might act as a haptene in suitable combination with a foreign protein carrier.

There is yet no precise knowledge of the chemical nature of the anterior pituitary hormones, or the principles of similar activity found in the urine of pregnancy, but it is known that they are relatively complex and thermolabile substances. This group includes the only hormone preparations which, hitherto, have been found to evoke the appearance in the blood of the inhibitory substances or 'antihormones'. They are as yet obtainable only in complex extracts, in which the essential hormones are probably associated with substances of a protein type and usually from a species foreign to the experimental animal in which the production of the antihormones is studied. Such a hormone, even if its structure is identical over a wide range of species, might conceivably act as a haptene, if suitably associated with a species-limited or organ specific carrier. A precipitating antibody formed in response to such a complex might give a precipitate in this case with the hormone-haptene, or, as in the case already mentioned of thyroxine in artificial combination, might neutralize the hormone activity without forming a precipitate, except when the hormone is linked to the antigenic carrier.

Tests *in vitro* for antihormones have so far been inconclusive, mainly on account of the fact that the antigens used have been relatively crude extracts of pituitary glands, urine, or serum, containing a multiplicity of antigens. Only relatively crude, qualitative methods have therefore been possible.

Complement fixation tests have been employed by Bachman [1935], who concludes that the complement-fixing and hormone inhibitory powers of antisera to extracts from urine of pregnancy do not run parallel. Similar conclusions are reached by Brandt and Goldhammer [1936]. Eichbaum and Kindermann [1935, 1936], using anterior pituitary extracts, conclude that the antiserum contains complement-fixing antibodies specific for the pituitary thyrotrophic hormone. They were, however, unable to demonstrate hormone-specific antibodies to urinary gonadotrophic extracts. Sulman [1937], using relatively crude pregnancy urine extracts, obtained complement fixation with antisera prepared against crude prolan, but a negative result when a highly purified extract (pure prolan) was used as antigen in the test. He concludes that pure prolan is not antigenic.

Using the ring test method, Twombly [1936] demonstrated precipitins with the serum of rabbits immunized to gonadotrophic extracts of pregnancy urine, and found that their appearance in the antisera coincided with that of the hormone-inhibiting substance.

The experiments here described were undertaken in the hope that

clearer evidence of the nature of the so-called antihormones, and parti-
cularly of their relation to the antibodies demonstrable by reactions *in
vitro*, might be obtained by using preparations of the highest activity
which purification could produce, and still capable of evoking the appear-
ance in the blood-serum of inhibitory substances or antihormones, when
given in a suitable series of injections.

For this reason, purified gonadotrophic extracts from pregnancy urine
were used. It was hoped that quantitative serological methods would
make it possible to determine whether the inhibitory power of a serum
was or was not dependent on its antibody content. If the antibody nature
of the inhibitory substance could be established by these methods, the
same antisera could then be used to investigate the immunological
importance of the hormone-active part of the antigen. If the hormone
acts as a specific haptene, or if antibodies are formed, which are specific
for the carrier in a hormone-antigen complex, the amounts of different
urinary extracts required for complete precipitation with the same
amount of antiserum would be expected to show an inverse relationship
to the biological activities of the extracts. In other words, activity as
hormone and activity as antigen ought to vary together.

<div align="center">METHODS</div>

Biological assay of extracts. These were kindly performed by Dr. I. W.
Rowlands. Several commercial preparations of the gonadotrophic hor-
mone of the urine of pregnant women were used. In addition, use was
also made of an extract from the urine of men. This extract was kindly
prepared by Organon Laboratory by a method identical with that used
in the manufacture of Pregnyl. The biological activity of these extracts
(see Table I) was determined by their ability to stimulate the ovaries of
the immature rat. Groups of 5–10 rats, weighing 40–50 g., were injected
subcutaneously, once daily for 5 days, with the extract dissolved in a
standard volume of 1 c.c. of distilled water. The animals were killed 24
hours after the last injection, the ovaries and uteri dissected, and, after
fixation in Bouin's fluid overnight, were weighed from 70% alcohol.

A dose-response curve (Fig. 1) was constructed from the extract
(Pregnyl, UP27), which was used in the production of the inhibitory
sera. From this curve the comparative activity of all other extracts was
determined by calculating the amount of each substance which is equiva-
lent to 1 mg. UP27 used as a standard for the purpose. The data are given
in Table I.

Biological activity of antisera. The activity of each serum was deter-
mined by its ability to inhibit the action, on the ovaries of the immature
rat, of a standard amount (0·05 mg.) of the extract UP27. Both extract

and antiserum were injected simultaneously, on opposite sides of the animal, once daily for 5 days, subsequent treatment being the same as that described for the assay of the extract. A group of 5 or 10 rats was used for each antiserum.
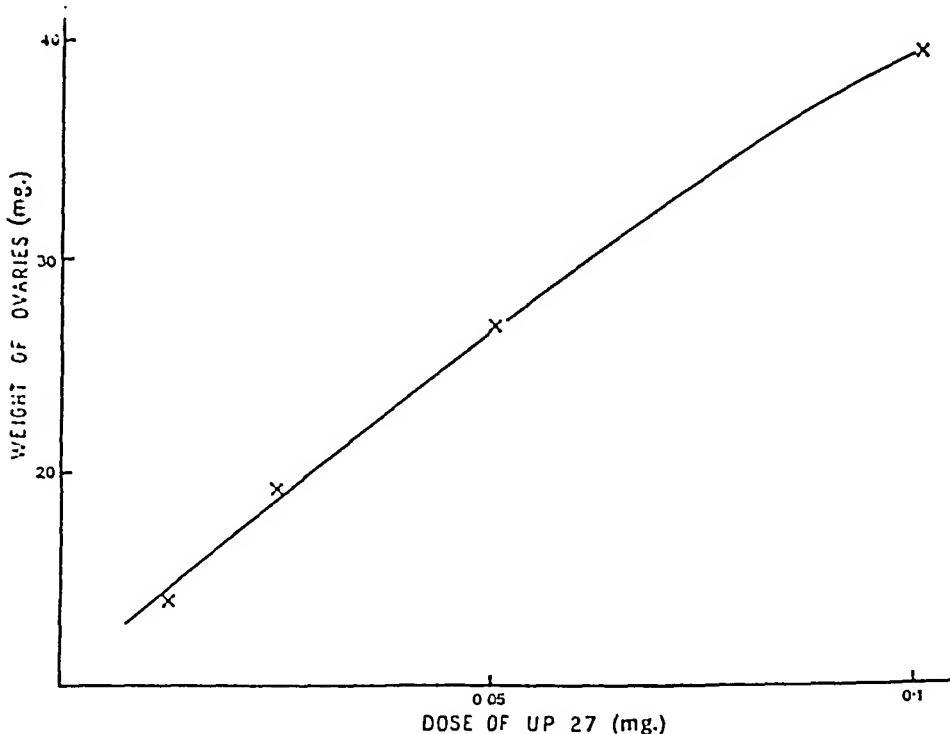
Fɪɢ. 1. Curve showing the average weights of ovaries in groups of rats given varying amounts of UP27 (Pregnyl)

The degree of inhibition which is observed can be calculated by translating the response obtained in terms of an amount of extract, by reference to the dose-response curve (Fig. 1). Subtraction of the amount so calculated from the quantity of extract injected gives the amount of hormone

Table I

Extract	Hormone activity on rat ovaries
Pregnyl (Organon) UP27	1·0
*Physox (Leo) UP24	0·17
*Follutein (Squibb) PU3	0·2
Gonan (British Drug Houses) UP16	0·3
Male Urine (Organon) MUG28	0·004
Prolan (Bayer) UP28	1·6

* These preparations were dilutions of the purified extract with 90 p.c. or more of lactose.
The hormone activity is expressed as the equivalent in mg. of UP27, of 1·0 mg. of each preparation used, all having been assayed by the same method.

inhibited by a known amount of antiserum. All the results are expressed as the amounts of extract (UP27) inhibited by 1 c.c. of antiserum.

Preparation of inhibitory sera. Each of ten rabbits (AUP11–20) was given daily subcutaneous injections of 2·5 mg. of an extract of pregnancy urine (Pregnyl, UP27) over a period of 7–8 months. The animals were bled after $3\frac{1}{2}$ months of immunization, and thereafter at monthly intervals. Injections of hormone were suspended for the two days preceding each bleeding.

The blood was allowed to clot at room temperature, when the serum was pipetted off and freed from red cells by vigorous centrifugation. After standing overnight at 0° C., any of the sera that were cloudy from precipitation of lipoids were cleared by passage through a Seitz filter. All were stored in the frozen state.

The second, fourth, and sixth bleedings (B, D, F) of five of the rabbits (11, 14, 15, 16, and 18) were tested separately. The remaining sera of each bleeding were pooled, making a mixed sample for each length of immunization. There were, therefore, 15 individual samples and 6 pools for investigation.

Precipitation reactions. When only a qualitative test for the presence of precipitins was required, ring tests were done; increasing dilutions of antigens in 0·1 c.c. volume were layered over an equal volume of undiluted antiserum, and the tubes examined at fixed intervals for the presence of rings. After standing for $1\frac{1}{2}$ hours, the tubes were shaken, and examined 6 to 12 hours later for the presence of opalescence or precipitates. In many cases a rough indication of the amount of precipitin present could be obtained by picking the tube with the coarsest particles at this time.

For the quantitive estimate of precipitins the optimal proportions method of Dean and Webb [1926] was employed. In view of the delicacy of the precipitates formed, it was important to use thoroughly cleaned, unscratched test-tubes of uniform diameter.

In earlier experiments, numerous technical difficulties were encountered. Thus antisera obtained after only a short period of immunization reacted only very slowly if the antisera were used in a five- or tenfold dilution. With higher concentrations of such antisera, precipitates were denser and formed more rapidly, but the small quantities of serum available made its use without dilution in quantitative tests impracticable. After more prolonged immunization, it became possible to use serum 1/2·5 or 1/5, but even then, in many cases, precipitates were so fine, and formed so slowly, that an optimal tube could be selected only with great difficulty, and in many instances 'first particulation' occurred in a wide zone of antigen concentrations.

In all the experiments reported here, antiserum was diluted 2·5 or 5

times. Of this dilution, 0·5 c.c. was mixed with an equal volume of antigen solution in each tube of the test. Rough tests, in which the concentration of antigen was halved in successive tubes, gave an approximate indication of the amount of precipitin present. These mixtures were kept at room temperature and examined at frequent intervals for 4–6 hours, and again after a further period of 12–24 hours. In this way it was found possible to determine the first zone of flocculation as well as any subsidiary zones, which often occurred later. In the subsequent finer tests, the amounts of antigen in successive tubes differed by as small a proportion as was compatible with accurate determination of an optimum. A fairly wide margin of antigen excess was allowed for in the fine tests. The tubes were observed for the first opalescence. An attempt was then made to determine the first flocculating tube, with the aid of a hand lens. This, owing to the delicacy of the precipitate, was not always possible, and the first flocculation was then determined by careful observation of the particles as they became visible to the naked eye. Even then it was sometimes impossible to decide with certainty in which of two or three tubes particulation occurred first. The selection of an optimal mixture was then based on the coarseness of the particles. All tests were kept under observation, at least until sedimentation of floccules in the optimal zone had commenced. The amount of antigen at optimal proportions with 1·0 c.c. of each antiserum was calculated from an average between the antigen concentrations in the first and second flocculating tubes of the fine test.

All the antigens used were tested also with two batches of pooled normal rabbit serum. Non-specific precipitation was found to occur, after 6 hours or more, only with the higher antigen concentrations.

The amounts of precipitin for human serum were similarly determined by rough and fine tests.

In the hope of increasing the speed of precipitation and the bulk of the precipitate, attempts were made to adsorb the antigen on finely divided particulate substances such as collodion particles, or colloidal carbon. In no case, however, was the result significantly different from that obtained with unadsorbed antigen.

RESULTS

Precipitation Reactions with Pregnyl

Results obtained with the precipitation reaction show that the urinary gonadotrophic extracts cannot be regarded as immunologically pure substances, in spite of the high degree of purity suggested by the activity per unit weight. The complex antigenic nature of the extracts is suggested, but not proved, by a second zone of flocculation. In most cases, where two zones occurred they appeared at widely different antigen con-

centrations, but in one or two instances they were closely adjacent, resulting in one wide range of flocculation. This probably accounts for the difficulties experienced in earlier experiments where, owing to slowness of precipitation, the multiplicity of zones was not noticed, and particles appeared to form simultaneously in four or more tubes of the rough test. Owing to the constancy with which the same zones occurred in these experiments, it was thought probable that the optimal zone was related to the same antigen throughout. The only apparent exceptions are the three sera of rabbit 14, where first particulation occurred over a range of antigen concentrations different from that obtained with the other sera. A typical example of a precipitation reaction is given in Table II.

Table II

Amount of Pregnyl in mg.	5	2·5	1·25	0·625	0·31	0·16	0·08	0·04	0·02	0·01	Saline control
Amount of Anti-serum in c.c.											
AUP18B	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1
10 minutes	—	—	0—	0	0	0—	0—	0=	—	—	—
25 ,,	—	—	0—	0vfP	0vfP	0—	0—	0—	—	—	—.
				Zone 1		Stop-gap	Zone 2				
45 ,,	—	—	0—	0vfP	0vfP	0	0vfP	0—	0=	—	—
1 hour	—	—	0—	0vfP	0vfP	0vfP	0vfP	0—vfP	0=	—	—
1½ ,,	—	0=	0—VfP	0fP	0fP	0fP	0fP	0—vfP	0=	—	—

Result of Rough Test showing two zones:
1st: 1·0 c.c.≡6·25—3·1 mg. Pregnyl
(with subsequent fine tests the optimal was determined at ±5·25 mg.)

2nd: 1·0 c.c.≡0·8 mg.

0=, very faint opalescence; 0—, slight opalescence; 0, opalescence; vfP, very fine particles; fP, fine particles. Stop-gap: Tube showing only persistent opalescence, interposed between two zones of flocculation.

Complex antigenic nature of Pregnyl. The presence of zoning alone cannot be regarded as proof of the presence of multiple antigens in the urinary extracts. According to Kabat and Heidelberger [1937], however, complexity of antigen can be shown by the presence of antigen or antibody in the supernatent fluid of 'equivalence zones', after removal of specific precipitate. To apply this test, five 10-c.c. quantities of a pooled serum (AUP, Pool B) were absorbed in weighed conical Monax centrifuge tubes, with varying amounts of UP27 dissolved in 10 c.c. of saline. The reagents were left in contact for 2 hours at 37° and overnight in the icebox. The precipitates were then centrifuged down, and the supernatant fluids drawn off. The precipitates were washed once in saline, and once in

distilled water. (No heat coagulable protein was present in the water after the second washing.) They were then dried to constant weight over phosphorus pentoxide in high vacuum. After the dry weights of the precipitates had been obtained, the total nitrogen of each was determined by the micro-Kjeldahl method. The absorbed sera were tested for free antigen, by layering 0·1 c.c. quantities of progressive dilutions over an equal volume of unabsorbed serum. The tests were observed for rings at 15-, 30-, and 60-minute intervals. No readings were taken after standing overnight owing to confusion arising from non-specific precipitation which occurs in higher antigen concentrations. For free antibody, ring tests were done, using the homologous antigen (Pregnyl). In addition, each absorbed sample was tested for its power to inhibit the action of a small test dose (0·025 mg.) of Pregnyl in immature rats. The results are recorded in Table III.

Table III

	Amount of Pregnyl added per c.c. anti-serum mg.	Weight of specific precipitate from 10 c.c. serum mg.	Nitrogen in total precipitate mg.	Free anti-gen	Free anti-body	Wt. of ovary after 0·04 c.c. absorbed serum +0·025 mg. Pregnyl mg.
1	4·0	2·3	0·54	+ +	—	23·5
2	*2·75	2·8	0·54	+ +	—	22
3	1·37	2·8	0·57	+	—	22
4	0·6	2·7	0·50	+	+	15
5	0·3	2·0	0·41	+	+	11
6	Control of unabsorbed antiserum and Pregnyl					15
7	Control of Pregnyl alone					19·3

*Amount at optimal proportions with the antiserum.

The presence of excess of antigen in all the tubes is in favour of the complex antigenic nature of Pregnyl. The results of the tests for excess of antibody shown in the table cannot be accepted with much confidence. Visible rings could not be obtained in control experiments with unabsorbed serum diluted 10 times. Traces of free antibody may therefore have been present in 1, 2, and 3, in insufficient amounts to be demonstrable by the test. The experiment was repeated, using an antiserum (AUP, Pool F) in which antibody content was higher and speed flocculation more rapid. A similar result was obtained as regards presence of antigen in the supernatant fluid of the tube precipitated at the optimal ratio, and, in addition, the presence of antibody, even in the tubes absorbed at antigen excess, was shown by the development of opalescence in such tubes with the ring test. It can therefore be concluded that the supernatant in the 'equivalence' tube contained both free antigen and antibody. Pregnyl must therefore contain more than one precipitinogen. The very small bulk of the precipitate obtained from 10 c.c. of the antiserum, whether at optimal

ratio or with antigen excess, shows further that only a small fraction of the total weight of urinary extract can be active as precipitating antigen in these experiments.

In these circumstances, a study of the results of such tests *in vitro* cannot be expected to give a definite answer to the question, whether the substance in the sera which inhibits the hormone is concerned in the visible reactions with precipitating antibodies. More than one precipitating antigen being present, and the supernatant fluid from an optimal reaction containing both antigen and antibody, the biological tests of such fluids would in any case be difficult to interpret. The results in Table III show further that the biological test has a very low quantitative accuracy. The biological tests on tubes 5 and 6, if taken at their face value, would indicate that the untreated antiserum has a lower inhibitory action on the preparation UP27 than it has after absorption with 0·3 mg. of that preparation per c.c.

Relations between Precipitin Content and the Inhibitory Action on Gonado-trophic Hormone

Comparison between the precipitin content of the antisera used, and their power to inhibit the effect of the homologous hormone on the ovary weight of immature rats. The results are summarized in Table IV. The figures demonstrate that no correlation exists between the precipitin content of the sera and their biological inhibitory power. In the earlier bleedings, A, B, and C, the results obtained with the precipitin reaction are higher. With bleeding D there is more apparent correlation, although the results of the biological tests are now slightly greater. The results with E and F antisera show an increasing discrepancy, the values obtained with the precipitin reaction remaining more or less constant, whilst the biological inhibitory power steadily increases.

The results are expressed graphically in Fig. 2. Each point represents an average value of all the results with antisera of each bleeding; thus the B, D, and F readings take into account also the results obtained with the individual bleedings of rabbits 11, 14, 15, 16, and 18. It is seen that the precipitin values for the individual bleedings of rabbit 14 are all lower than in the corresponding bleedings of the other rabbits. The difference can probably be explained by the fact that first flocculation in the serum samples of rabbit 14 occurred in a zone which, for the sera of the other rabbits, was only subsidiary. If, however, in constructing Fig. 2, the precipitins in the sera 14 B, D, and F are not included, the shape of the curve for precipitins to Pregnyl is not significantly altered. A curve for the amount of precipitin for human serum is also shown, and can be seen to run parallel to that for the precipitins of UP27. As will be seen

later, absorption with human serum does not remove all the precipitins present in the antisera.

The power of antipregnyl (UP27) sera to precipitate other known gonado-trophic extracts of pregnancy urine. The preparations used are given in Table I. All these extracts reacted to antipregnyl sera with the optimal proportions method. With Physex and Follutein zoning was less frequent and not always clearly defined, but the determination of optima in fine tests was no more difficult than with Pregnyl. They were all tested with two or three samples of the pooled sera A, B, and C. Results are tabulated in Table V.

Table IV

Serum	Biological	Precipitin UP27	Precipitin H serum	Zones
Pool A	1·75	2·75	0·0032	—
,, B	2·0	2·75	0·0031	—
,, C	2·8	5·25	0·0051	—
,, D	5·6	4·5	0·0045	—
,, E	9·0	4·5	0·0037	—
,, F	10·0	5·6	0·0037	—
11 B	1·7	5·0	0·0017	—
14 B	1·75	0·87	0·0017	at 3·25 mg.
15 B	3·5	4·5	0·0059	,, 0·8 mg.
16 B	1·85	3·6	0·0006	? ,, 0·8 mg.
18 B	1·65	5·6	0·0055	,, 0·8 mg.
11 D	2·8	6·5	0·0022	,, 0·8–1·6 mg.
14 D	3·7	1·12	0·0027	—
15 D	7·4	8·75	0·0085	—
16 D	3·7	5·6	0·0022	,, 0·8–1·6 mg.
18 D	4·7	8·0	0·0044	—
11 F	5·0	4·5	—	,, 0·8 mg.
14 F	5·6	2·25	—	—
15 F	11·2	9·0	—	—
16 F	7·4	4·5	—	Very wide optimal zone
18 F	4·6	7·5	—	—

Biological activity refers to the number of milligrams of UP27 inhibited by 10 c.c. of the serum in the rat test. Precipitin UP27 = number of milligrams of the hormone at optimal proportions with 1·0 c.c., and Precipitin H serum = number of c.c. of human serum at optimal proportions with 1·0 c.c. The same batch of human serum was used throughout.

Table V

Antiserum	Pregnyl		Physex		Follutein		Gonan		Male Urine	Prolan	
AUP, Pool A	2·75	2·75	6·85	1·14	1·75	0·35	0·6	0·18	4·5	0·018	0·225	0·36
AUP, Pool B	2·75	2·75	6·25	1·04	not tested		not tested		3·25	0·013	0·225	0·36
AUP, Pool C	5·25	5·25	6·8	1·13	1·4	0·3	0·6	0·18	3·1– 6·2	0·012– 0·024	0·25	0·40

The first column under the name of each extract refers to the amount in milligrams of the extract at optimal proportions with 1·0 c.c. of the antiserum, and the second column represents the equivalent hormone activity of that value in terms of UP27. They were calculated from the values of the various preparations, as given in Table III.

The amount of an extract at optimal proportions with 1·0 c.c. of the anti-serum is therefore not related to the hormone content of the extract.

Absorption Experiments

To show the effect of removing antibodies to antigens characteristic of the urine but not of the hormone preparation on the inhibitory power of the antisera, antiserum AUP, Pool C, was absorbed at optimal proportions
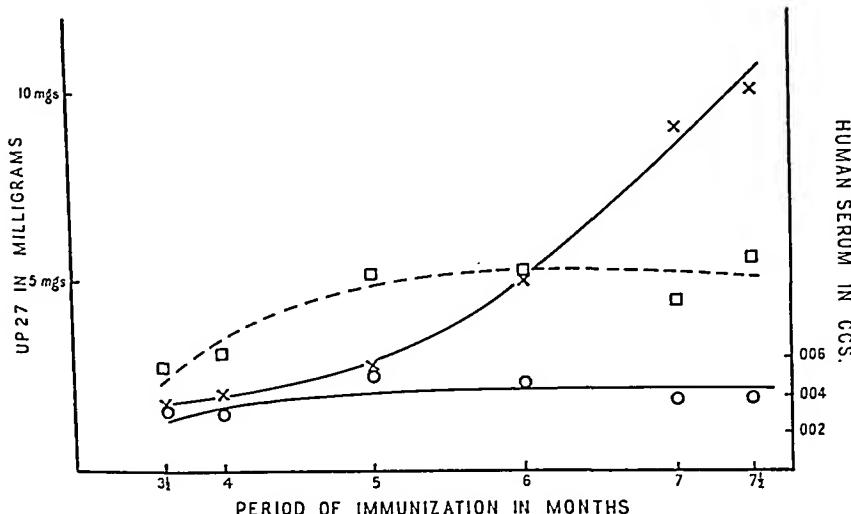
FIG. 2. □----□ shows the amount in milligrams of UP27 at optimal proportions in the precipitation reaction with 1·0 c.c. of antiserum; ×——× the amount of UP27 inhibited by 1·0 c.c. in the rat test; and ○——○ the amount in c.c. of human serum at optimal proportions with 1·0 c.c. of the antiserum. The scale for the amount of human serum precipitated is shown on the right, and bears no relation to that for UP27; and curve ○——○ is included in the same figure as the other two for convenience only.

with normal human serum (0·052 c.c. in 2·0 c.c. saline added to 10 c.c. of antiserum) for 2 hours at 37° and 2 days in the icebox. The absorbed serum, when tested biologically, was able to neutralize the action of 2·64 mg. hormone per c.c., which does not differ significantly from the amount neutralized by the unabsorbed serum (2·8 mg. per c.c.).

The total weight of the precipitate produced by human serum from 10 c.c. of antiserum was 1·5 mg. Precipitins for human serum were now absent, but precipitins for Pregnyl could still be demonstrated by ring tests.

Absorption of urinary precipitins from antiserum AUP, Pool B, by contact with an extract of the urine of males (MUG28) at the optimal ratio, produced a specific precipitate of 2·8 mg. from 10 c.c. of antiserum. Such absorbed serum no longer contained a measurable amount of pre-cipitins for Pregnyl, but was still able to inhibit the action of Pregnyl when tested in rats, though the inhibitory power had been reduced by

approximately one-half. Similarly, absorption by the extract of the urine of males at optimal proportions of a different serum sample, AUP, Pool F, led to complete loss of precipitins for the extract of the urine of males and for Pregnyl, but the antiserum still retained one-third of its original inhibitory power for Pregnyl. One cubic centimetre of this antiserum neutralized the activity of 2·8 mg. Pregnyl after absorption as compared with $\pm$ 10 mg. before absorption.

DISCUSSION

While the experiments here reported have not given the clear answer which it was hoped to obtain concerning the nature of the antihormones, they afford useful information in several directions with regard to the antigens present in a purified gonadotrophic urinary extract, and the antibodies to them, which are formed in response to the long course of daily injections used to produce antihormones inhibiting the gonadotrophic activity.

An antiserum so prepared against one such extract, Pregnyl, has been shown to contain precipitins, demonstrable by quantitative methods, not only with Pregnyl itself as the combining antigen, but also with the most highly purified urinary gonadotrophins available, as judged by the relation between weight and activity. This finding contrasts with that of Sulman, who failed to obtain complement fixation with Prolan as antigen. The fact, however, that precipitins can thus be demonstrated in a serum with antihormone activity does not identify the antihormone as a precipitin, or the hormone itself, or its complex, with a carrier, as a precipitinogen. The amount of specific precipitate obtained at optimal proportions is so small that only a small fraction of the material of the urinary extract can be concerned as an antigen in its formation; and this small fraction might consist entirely of substances containing no hormone, even with the most highly purified and active extracts yet available.

It has long been known that normal urine contains colloidal, non-dialysable constituents, which can act as precipitinogens [Doerr and Pick, 1914; Landsteiner and v. Eissler, quoted from Rhein, 1913] and render guinea-pigs actively anaphylactic [Uhlenhuth and Haendel, 1910; Rhein; Uhlenhuth and Seiffert, 1929]. According to Savare [1907], the amount of non-dialysable material in normal urines, and in urines from the later stages of pregnancy, is approximately the same. It is very probable, then, that the precipitins which have been found in the antiserum produced by long immunization against a urinary gonadotrophic extract were largely of the same kind as those which Doerr and Pick obtained against constituents of normal urine. Some light is thrown upon the relation of the antihormone to the precipitins by certain of the data recorded in this

paper. The quantitative precipitation reactions showing the presence of zones, and the incompleteness of the precipitation of either antigens or antibodies from a mixture at optimal proportions, show clearly that several antigens were present in the extract used for immunization. When the quantity required to produce optimal precipitation, with a fixed amount of an antiserum, is determined for each of a series of gonadotrophic urinary extracts of varying degrees of purification, the quantities so determined exhibit a wide range of different gonadotrophic activities. Such data, however, merely prove that the extracts contain, in varying proportions, precipitinogens which have no hormone action; they leave open the question whether the hormone, as present in the extract, is wholly or partly associated with a precipitinogen. Absorption experiments show that an extract of the urine of males removes precipitins from an antiserum as completely as a gonadotrophin from pregnancy urine (Pregnyl), and greatly reduces the antihormone activity. This, however, may only mean that the precipitinogenic colloids are the same in the urine of males as in that of pregnant females, and that a part of the gonadotrophic hormone present in the latter is associated with a normal precipitinogenic colloid to form a complex antigen.

The fact that the antiserum, after absorption with the extract of the urine of males, still possesses antihormone activity, though a weakened one, might be due to another type of antibody evoked by an antigen in which the hormone plays the part of a specific haptene and is associated with another urinary colloid.

Comparison of the rate of appearance of antigonadotrophic activity in the serum, during the long course of immunization, with the rate of appearance of precipitins for the constituents characteristic of urine, and for normal human proteins, determined in both cases by quantitative precipitation reactions, shows that the precipitins rise to a maximum at a stage when the antigonadotrophic action is still increasing, and that the latter continues to rise steeply, while no further increase of precipitins can be detected. This seems to indicate, like the other evidence, that the antigonadotrophic substance has not the nature of a precipitin. If the hormone were acting as a haptene attached to a precipitinogenic antigen, the maximum antigonadotrophic action might be expected to appear with the maximal precipitin content of the serum. Even such a deduction, however, cannot be made with certainty. If the hormone was chiefly associated with one of the urinary precipitinogens, which was either very small in amount or weak and delayed in its antigenic action, the antigonadotrophic action might continue to rise steeply at a stage of the immunization when no measurable addition was being made to the precipitin content.

There is further the possibility that the hormone may be predominantly associated with a urinary constituent, which acts as an antigen, bu evokes an antibody of a kind yielding no actual precipitate under th particular conditions of the tests. In either case it is clear that there ar possibilities of explaining the antigonadotrophic action, without assum ing that it is not due to a true antibody, or, if it be due to an antibody that the free hormone, if it could be obtained, would act as a complete antigen.

It is also clear, however, that the preparations of the urinary gonado trophin yet available are still so complex in their antigenic structure that they are unsuited for study by accurate serological methods with anti sera obtained after a course of injections so long and so frequent as that required to obtain a strong antigonadotrophic action. Definite evidence as to the nature of the antihormone cannot be obtained by such methods, unless a preparation should become available which is strong in hormone activity and makes a much nearer approach to immunological purity.

SUMMARY

Antisera to an active gonadotrophic extract of pregnancy urine contain precipitins demonstrable with a variety of urinary extracts, independent of their hormone activity. The amounts of precipitins present could be determined by optimal proportions.

Precipitin content and antigonadotrophic power showed no constant relationship to each other.

Pregnancy urine extracts contain multiple antigens.

Absorption of precipitins by an extract of the urine of males resulted also in the removal of all precipitins for urinary gonadotrophic extracts, but only partly reduced the biological inhibitory power. On the other hand, absorption of precipitins for human serum only slightly reduced the precipitins for urinary extracts, and left the biological inhibitory power completely unimpaired.

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THE RATE OF APPEARANCE OF ANTI-LUTEIN-IZING ACTIVITY IN THE SERUM OF RABBITS INJECTED WITH EXTRACT OF OX PITUITARY GLAND

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IN recent experiments it was found possible to change the qualitative nature of the response of the ovaries of the immature rat to injections of a gonadotrophic extract of gelding pituitary gland by the simultaneous administration of an antigonadotrophic serum obtained by immunizing rabbits with an extract of ox pituitary gland [Rowlands, 1938]. When the dose of antiserum was carefully adjusted, the luteinizing activity of the extract was inhibited completely and selectively, and the ovaries of the immature test rats responded only by follicular growth. This purely follicle-stimulating effect could not be produced by an amount of extract of gelding pituitary gland alone, which was capable of causing a similar increase in ovarian weight.

This result was tentatively explained as being due to the facts that extracts of gelding pituitary gland have much follicle-stimulating activity and little luteinizing activity, whereas extracts of ox pituitary gland have much luteinizing and little follicle-stimulating activity, so that an antiserum to the latter may be expected to have much anti-luteinizing activity and little anti-follicle-stimulating activity. Such an antiserum, interacting in suitable amounts with an extract having mostly follicle-stimulating activity, would presumably neutralize the small amount of luteinizing activity and leave a residue of purely follicle-stimulating activity. The effectiveness of an antiserum in making such a differential neutralization of an extract is obviously dependent on its exact qualitative properties. Since there is evidence that the qualitative properties of antigonadotrophic sera are influenced by the period of immunization [Rowlands, 1939], the investigation recorded here was made to trace the rate of appearance of anti-luteinizing activity in the serum of rabbits injected with extracts of ox pituitary gland, and to determine the stage of immunization which gives a maximum concentration of anti-luteinizing activity, relative to anti-follicle-stimulating activity.

METHODS

Six adult female rabbits (ATH 114–19) were given daily subcutaneous injections of 25 mg. of an extract of ox pituitary gland (AP14B) suspended in 5 c.c. of distilled water. They were bled from the marginal ear vein at weekly intervals after an initial period of 10 days, and on each occasion the samples of serum (Serum B) obtained from the individual animals were pooled. The rabbits were bled finally under ether anaesthesia, after 12½ weeks of continuous daily injections, and 200 c.c. of serum were obtained.

Each sample of serum was assayed by its capacity to inhibit selectively the luteinizing activity of the extract of gelding pituitary gland (AP70B). Each week 4 to 5 groups of rats, each consisting of five animals, were injected with a constant amount of extract (0·5 mg. daily), and with varying amounts of serum. The method of assay was similar to that used by Rowlands [1938]. Serial sections were cut, one in five mounted, and stained by the histological technique previously described. The criterion of follicle stimulation was taken as the presence of large follicles without any luteinization of the cells of the membrana granulosa. The results are expressed as the percentage number of animals in a group whose ovaries show a complete absence of luteinization. The presence, in one animal of a group of five, of a single corpus luteum, or even of some slight luteinization of the membrana granulosa of one follicle in an ovary, can accordingly change the index by 20%. This occurred occasionally in the experiments recorded below, so that the percentage figure may give an unduly low estimate of the degree to which the luteinizing effect was inhibited by the serum.

RESULTS

The data relating to the quantitative and qualitative effects of the rabbit sera obtained after different periods of injections, on the response of the ovaries of the immature rat to the constant amount (2·5 mg.) of the extract of gelding pituitary gland (AP70B), are shown in Table I and Fig. 1. This standard amount of extract increases the weight of the ovaries of the young rat from the normal of 10 mg. to 54 mg., and increases the weight of the uterus from 20 mg. to 65 mg. The simultaneous injection of normal rabbit serum increased the quantitative response in the ovaries to this amount of extract AP70B (Table I), but the amount of such serum injected failed, in all rats except one, to give any inhibition of the luteinizing activity of the extract [Rowlands, 1938].

2·5 c.c. and 5 c.c. of serum obtained after a period of injections lasting 1½ weeks produced an augmentatory effect on the quantitative response of the ovary to the extract AP70B, which appeared to be significantly

Table I

The effect on the reproductive organs of the immature rat of varying amounts of Serum B, taken at weekly intervals during immunization, on a constant amount of gelding pituitary extract (APPOB). ovs. = average weight of ovaries; ut. = average weight of uterus; No Lut. = percentage of animals whose ovaries show absence of luteinization. When luteinization is completely inhibited the weight of the ovaries is given in Clarendon type.

Period of immunization after which serum was obtained (weeks)	Amount of Serum (c.c.)																	
	0·25			0·5			1·0			1·25			2·5			5·0		
	ovs.	ut.	No Lut.	ovs.	ut.	No Lut.	ovs.	ut.	No Lut.	ovs.	ut.	No Lut.	ovs.	ut.	No Lut.	ovs.	ut.	No Lut.
	mg.	mg.	%	mg.	mg.	%	mg.	mg.	%	mg.	mg.	%	mg.	mg.	%	mg.	mg.	%
0	—	—	—	—	—	—	—	—	—	69	88	20	76	78	0	76	71	0
1½	—	—	—	—	—	—	—	—	—	57	87	40	95	65	0	116	82	0
2½	—	—	—	—	—	—	—	—	—	92*	91	25	87	94	0	60	107	60
3½	—	—	—	—	—	—	—	—	—	66	84	40	57	138	60	45	103	100
4½	—	—	—	—	—	—	—	—	—	45*	102	50	37	100	80	27	82	100
5½	—	—	—	64	84	20	59	106	60	40	100	40	37	94	80	37	94	80
7½	—	—	—	84	91	100	62	109	80	—	—	—	45	95	100	36	114	100
8½	—	—	—	72	83	80	44	112	100	64	117	80	42	105	100	26	93	100
9½	—	—	—	56	94	100	51	98	100	—	—	—	35	104	100	31	92	100
10½	95	75	0	72	76	60	64	100	80	—	—	—	42	106	100	21	101	100
11½	69	97	40	45	106	100	41	119	80	—	—	—	25	112	80	18	62	40
12½	51	96	80	55	90	60	63	108	80	—	—	—	26	116	80	20	92	100

* 4 rats in this group.

greater than that given by normal rabbit serum. No qualitative effect on the response was observed, however, with serum taken at this early stage of immunization, as corpora lutea were present in the ovaries of all these rats. Assays carried out with 2·5 c.c. and 5 c.c. of serum taken at
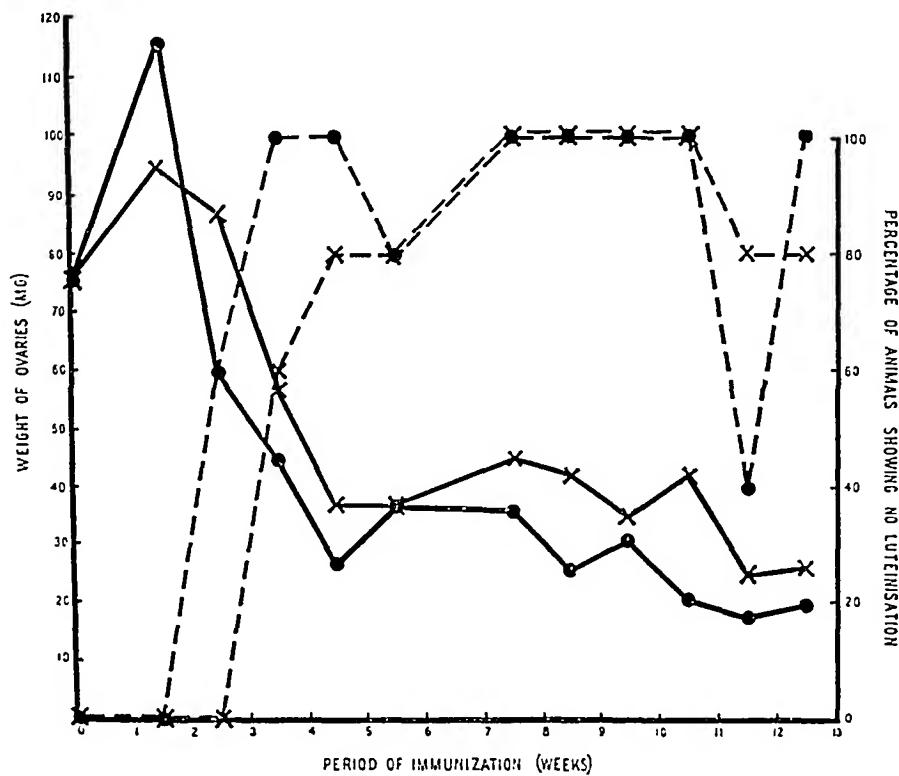
Fig. 1. The effect, on 2·5 mg. of the extract AP70B, of 2·5 c.c. and 5 c.c. of serum of rabbits (Serum B) obtained at weekly intervals during the course of injection with extract of ox pituitary gland (AP15B).

    X——X = effect of 2·5 c.c. of serum on weight of ovaries.
    X---X = effect of 2·5 c.c. of serum on luteinization.
    ●——● = effect of 5 c.c. on weight of ovaries.
    ●---● = effect of 5 c.c. on luteinization.

$2\frac{1}{2}$, $3\frac{1}{2}$, and $4\frac{1}{2}$ weeks from the beginning of the injection period show that the quantitative response in the ovaries becomes less, and that during the same time there is a progressive increase of the effect of the serum in suppressing luteinization. When 5 c.c. of serum taken after $3\frac{1}{2}$ weeks was injected, the average weight of the ovaries was 45 mg.; they all contained numerous large follicles showing no trace of luteinization. It has previously been shown that half the standard amount of extract received by these rats causes extensive luteinization with a smaller ovary weight, so

that the effect produced with the antiserum cannot be explained as being the result of a partial, non-selective neutralization of the activities of the extract as a whole. A smaller amount of serum (1·25 c.c.), from samples taken during the same period, produced a less marked effect on both the quantitative and the qualitative response.

Fig. 1 shows that the injection of 2·5 c.c. or 5 c.c. of serum from blood taken between 4½ and 12½ weeks caused only slight additional change in the quantitative and qualitative response of the ovaries to 2·5 mg. of the extract AP70B. The ovaries of rats injected with 2·5 c.c. of serum obtained at 7½–10½ weeks weighed about 40 mg. and contained only numerous large follicles; the uteri were in the greatly distended condition typical of oestrus in the normal animal. With serum obtained from the rabbits during this later period of immunization a similar selective neutralization of the luteinizing activity of the extract of gelding pituitary gland was obtained with even smaller dosage of the serum. At 9½ weeks only 0·5 c.c. of serum was required to neutralize completely the luteinizing activity of the extract, without affecting the quantitative response, but after 10½ weeks the selective anti-luteinizing activity of the serum appeared to be less.

SUMMARY

The serum of rabbits injected daily with a gonadotrophic extract of ox pituitary gland inhibits selectively, in immature rats, the luteinizing activity of a gonadotrophic extract of gelding pituitary gland [Rowlands, 1938]. The rate of appearance of this selective anti-luteinizing activity has now been investigated, by the examination of samples of serum obtained, at weekly intervals, during the period of injection of the rabbits with the extract of ox pituitary gland. The results (Fig. 1, Table I) show that the amount of anti-luteinizing activity present in the serum increases up to about 7 weeks after the commencement of immunization, is constant during the next 2 weeks, and, thereafter, falls somewhat.

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FURTHER OBSERVATIONS ON THE PRO-GONADOTROPHIC AND ANTITHYROTROPHIC ACTIVITY OF ANTISERA TO EXTRACTS OF THE ANTERIOR PITUITARY GLAND

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It was observed by Collip [1937] and Thompson [1937] that the serum of a sheep which had received a course of injections of sheep pituitary extract augmented the gonadotrophic activity of the same extract on the ovaries of the immature rat. Similar results were obtained by Rowlands [1938], who also showed that the serum of a sheep which had been treated in a similar manner, when injected into immature rats, had neither an augmentatory nor an inhibitory action on the gonadotrophic activity of extracts prepared from the pituitary glands of other species. However, the serum from a goat which had been injected with an extract of pig pituitary gland augmented the gonadotrophic action of the extract on the ovaries of the immature rat, but at the same time exerted an inhibitory effect on the ovulation-producing capacity of the same extract in the oestrous rabbit. In addition, the serum augmented the action, on the ovaries of the immature rat, of extracts of ox and sheep pituitary glands, but inhibited those of horse and human pituitary glands, and also those of the urine of pregnant women and those of the serum of pregnant mares.

The experiments described below were designed to trace the rate of appearance of the augmentatory factor in the serum of a goat receiving daily injections of an extract of pig pituitary gland, and, at the same time, to ascertain its relation to the rate of appearance of the antigonadotrophic and antithyrotrophic properties. Further data on the nature of augmentatory or pro-gonadotrophic activity were obtained from tests carried out on the serum of a dog injected with an ox pituitary extract containing both gonadotrophic and thyrotrophic activity.

METHODS

Animals immunized. (1) An adult bitch (Dog 1) was injected subcutaneously daily for 10 weeks, from 12.4.37 to 21.6.37, and for 5½ weeks, from 28.6.37 to 5.8.37, with 10 c.c. of a Seitz-filtered solution containing

2 mg./c.c. of an extract (AP32D₁) of ox pituitary gland. The thyrotrophic and gonadotrophic activities of this extract are shown in Tables I and II respectively. A sample of blood was taken from the jugular vein at weekly intervals after a period of 6 weeks from the start of the experiment.

(2) A goat (Goat 6) was injected in the same manner daily for 10 weeks, from 10.9.37 to 19.11.37, with 100 mg. of an extract of pig pituitary gland (AP53D), the gonadotrophic and thyrotrophic activities of which are also shown in Tables I and II. Samples of serum were obtained by bleeding the animal at fortnightly intervals.

Assays

(1) *Antithyrotrophic activity.* The sera were tested for their capacity to inhibit the thyrotrophic action of the extracts used for immunization. Assays were carried out on groups of 5–10 immature female guinea-pigs, weighing about 200 g., by the method of Rowlands and Parkes [1936]. The results are expressed as the increase in weight of the thyroid glands in response to the simultaneous injection of the extract and serum once daily for 5 days.

(2) *Pro-gonadotrophic activity.* The sera were examined for their capacity to augment the action of gonadotrophic extracts on the ovaries of the immature rat. The serum of Goat 6 was injected into rats, together with the extract (AP53D) with which the goat was immunized. The serum of Dog 1 was injected into rats, together with a pyridine extract of ox pituitary gland (AP15B) and also with a commercial preparation of pig pituitary gland—Ambinon (AP51). The method of assay has been described previously [Rowlands, 1938]. All injections were made subcutaneously, and the two substances were administered separately, on opposite sides of the animal. Augmentation is measured by calculating the number of times the ovarian weight response is increased, above the normal weight of 10 mg., by the simultaneous injection of the serum. The augmentation index can therefore be expressed as:

$$\frac{\text{Increase in weight of ovaries produced by extract} + \text{serum}}{\text{Increase in weight of ovaries produced by extract alone}}.$$

RESULTS

Antithyrotrophic activity of the sera. The results of the assays for antithyrotrophic activity on the different samples of the two sera are shown in Table I. The injection of 5 mg. of extract AP32D₁ caused an increase of 26 mg. in the weight of the thyroid glands of a group of guinea-pigs. The simultaneous injection of 5 c.c. of Dog 1 serum, from blood samples

obtained after 8 and 9 weeks' injection, failed to depress this response. In both these tests a slightly greater response in the thyroid glands was elicited than when the extract was given alone. This same extract has also shown little power to produce antithyrotrophic activity in the serum of rabbits [Rowlands and Young, 1939].

The serum of Goat 6, after 2 and 4 weeks' injection, contained no antithyrotrophic activity (Fig. 1). After 6 weeks, a total of 5 c.c. of the serum reduced the response of the thyroid gland to AP53D by about one-half; after 8 weeks' injection the antithyrotrophic activity of the serum was increased nearly 4 times. The rate of appearance of this inhibitory substance is similar to that which occurs in rabbits injected with extracts of ox pituitary gland [Rowlands and Parkes, 1936].

Table I. *The antithyrotrophic activity of the serum of Dog 1 and Goat 6*

Serum	Time of bleeding after first injection Weeks	Amount of serum injected c.c.	Extract injected to test animal Description	Amount mg.	Increase in weight of thyroid glands mg.
—	—	—	Ox pituitary AP32D$_1$	5	26
Dog 1	8	5	,, ,,	5	31
,,	9	5	,, ,,	5	31
—	—	—	Pig pituitary AP53D	5	22
Goat 6	2	5	,, ,,	5	24
,,	4	5	,, ,,	5	23
,,	6	5	,, ,,	5	12
,,	8	2·5	,, ,,	5	7

Pro-gonadotrophic activity of sera. The serum obtained from Dog 1 was tested for its capacity to augment the activity of ox and pig pituitary extract on the ovaries of the immature rat, as shown in Table II. Pituitary extracts from both these species are relatively inactive in causing ovarian enlargement in the immature rat. A total dose of 50 mg. of the extract of ox pituitary gland, AP15B, was required to produce ovaries weighing 15 mg., the weight of the ovaries of the normal rat of the same body-weight being 10 mg. The ovaries are only slightly stimulated; there is little follicular growth. In some follicles the membrana granulosa becomes precociously luteinized and forms a small solid corpus luteum, which encloses the ovum, whereas in others the membrana granulosa remains normal.

In the test rat the injection of 5 c.c. of Dog 1 serum, obtained after 5 weeks of daily injections, produced a fivefold augmentation of the effect on the ovary of the extract of ox pituitary gland. The serum obtained after 10 weeks of injections augmented the response to about the same extent. The ovaries of these rats contained numerous large corpora lutea, indicating that a greater amount of follicle stimulation had occurred

previous to the onset of luteinization. The uterus was stimulated and greatly enlarged and the vagina of each rat was open.

Two other samples of serum, obtained 11 and 14 weeks after the beginning of the first course of injections, were injected into rats simultaneously with an extract of pig pituitary gland (AP51). The quantitative response elicited in the ovaries of the immature rat was augmented fivefold by the simultaneous injection of 5 c.c. of each serum. The combined effect of serum and extract included the formation of large numbers of corpora

Table II. *Pro-gonadotrophic activity of serum of Dog 1 and Goat 6*

Serum	Time of bleeding after 1st injection Weeks	Total amount of serum injected to rats c.c.	Extract injected		Average weight of		Augmentation index
			Description	Amount mg.	Ovaries mg.	Uterus mg.	
—	—	—	Ox pituitary AP15B	50	15	22	—
Dog 1	5	5	,, ,,	,,	35	44	5
,,	6	5	,, ,,	,,	25	48	3
,,	10	5	,, ,,	,,	32	52	4·4
—	—	—	Pig pituitary AP51	25	22	25	—
Dog 1	11	5	,, ,,	,,	71	71	5·1
,,	14	5	,, ,,	,,	67	57	4·7
—	—	—	Pig pituitary AP53D	25	22	28	—
Goat 6	2	5	,, ,,	,,	49	49	3·2
,,	4	5	,, ,,	,,	56	66	3·8
,,	6	5	,, ,,	,,	80	52	5·8
,,	8	5	,, ,,	,,	64	47	4·5
,,	10	5	,, ,,	,,	54	42	3·7

lutea in the ovaries, together with uterine stimulation and vaginal opening. It is seen, therefore, that this serum retains its full pro-gonadotrophic activity for a considerable period of time.

Each sample of serum from Goat 6 was tested simultaneously for antithyrotrophic and pro-gonadotrophic activity (Fig. 1). It is seen that this serum possessed the power to augment the gonadotrophic action of the extract AP53D on the ovaries of the rat. This effect is demonstrable in the serum taken after 2 weeks of injections, when the response is augmented threefold, and reaches a maximum (a sixfold augmentation) after an injection period of 6 weeks. It is observed that at this time (6 weeks) the serum has only a weak antithyrotrophic action. Subsequently, and corresponding to the period of increasing antithyrotrophic activity, there is a decrease in the pro-gonadotrophic activity of the serum; at 10 weeks the response is less than fourfold. It is possible that, had the injections been continued for a longer period, this action of the serum would have completely disappeared and the serum might, ultimately, have

shown antigonadotrophic activity, as demonstrated by Collip [1937] and
Thompson [1937]. Unfortunately it was not possible to continue these
experiments farther.

DISCUSSION

The data presented in this paper confirm the earlier observations
described by me [Rowlands, 1938], namely, that antisera to gonadotro-

FIG. 1. The development of antithyrotrophic and pro-gonadotrophic activity in the
serum of Goat 6.

X = weight of ovaries of rats in response to the injection of 5 c.c. of serum and 25 mg. of
extract AP53D.

● = increase in weight of thyroid glands of guinea-pigs in response to the injection of
5 c.c. of serum and 5 mg. of extract of AP53D. The response of the thyroid glands
to 5 mg. of extract and 2·5 c.c. of the serum, taken after 8 weeks of injections, was
only 7 mg.

phic extracts of the anterior pituitary gland of certain species augment
the action of some gonadotrophic extracts on the ovaries of the immature
rat. I suggested that the mechanism of this pro-gonadotrophic activity
depended on a partial differential neutralization of the extract. It has
been found so far that augmentatory sera have been produced only in

previous to the onset of luteinization. The uterus was stimulated and greatly enlarged and the vagina of each rat was open.

Two other samples of serum, obtained 11 and 14 weeks after the beginning of the first course of injections, were injected into rats simultaneously with an extract of pig pituitary gland (AP51). The quantitative response elicited in the ovaries of the immature rat was augmented fivefold by the simultaneous injection of 5 c.c. of each serum. The combined effect of serum and extract included the formation of large numbers of corpora

Table II. *Pro-gonadotrophic activity of serum of Dog 1 and Goat 6*

Serum	Time of bleeding after 1st injection Weeks	Total amount of serum injected to rats c.c.	Extract injected Description	Amount mg.	Average weight of Ovaries mg.	Uterus mg.	Augmentation index
—	—	—	Ox pituitary AP15B	50	15	22	—
Dog 1	5	5	,, ,,	,,	35	44	5
,,	6	5	,, ,,	,,	25	48	3
,,	10	5	,, ,,	,,	32	52	4·4
—	—	—	Pig pituitary AP51	25	22	25	—
Dog 1	11	5	,, ,,	,,	71	71	5·1
,,	14	5	,, ,,	,,	67	57	4·7
—	—	—	Pig pituitary AP53D	25	22	28	—
Goat 6	2	5	,, ,,	,,	49	49	3·2
,,	4	5	,, ,,	,,	56	66	3·8
,,	6	5	,, ,,	,,	80	52	5·8
,,	8	5	,, ,,	,,	64	47	4·5
,,	10	5	,, ,,	,,	54	42	3·7

lutea in the ovaries, together with uterine stimulation and vaginal opening. It is seen, therefore, that this serum retains its full pro-gonadotrophic activity for a considerable period of time.

Each sample of serum from Goat 6 was tested simultaneously for antithyrotrophic and pro-gonadotrophic activity (Fig. 1). It is seen that this serum possessed the power to augment the gonadotrophic action of the extract AP53D on the ovaries of the rat. This effect is demonstrable in the serum taken after 2 weeks of injections, when the response is augmented threefold, and reaches a maximum (a sixfold augmentation) after an injection period of 6 weeks. It is observed that at this time (6 weeks) the serum has only a weak antithyrotrophic action. Subsequently, and corresponding to the period of increasing antithyrotrophic activity, there is a decrease in the pro-gonadotrophic activity of the serum; at 10 weeks the response is less than fourfold. It is possible that, had the injections been continued for a longer period, this action of the serum would have completely disappeared and the serum might, ultimately, have

shown antigonadotrophic activity, as demonstrated by Collip [1937] and Thompson [1937]. Unfortunately it was not possible to continue these experiments farther.

DISCUSSION

The data presented in this paper confirm the earlier observations described by me [Rowlands, 1938], namely, that antisera to gonadotro-

FIG. 1. The development of antithyrotrophic and pro-gonadotrophic activity in the serum of Goat 6.

X = weight of ovaries of rats in response to the injection of 5 c.c. of serum and 25 mg. of extract AP53D.

● = increase in weight of thyroid glands of guinea-pigs in response to the injection of 5 c.c. of serum and 5 mg. of extract of AP53D. The response of the thyroid glands to 5 mg. of extract and 2·5 c.c. of the serum, taken after 8 weeks of injections, was only 7 mg.

phic extracts of the anterior pituitary gland of certain species augment the action of some gonadotrophic extracts on the ovaries of the immature rat. I suggested that the mechanism of this pro-gonadotrophic activity depended on a partial differential neutralization of the extract. It has been found so far that augmentatory sera have been produced only in

response to continual injections of gonadotrophic extracts of the pituitary glands of sheep, pig, and ox. Extracts from these three species are, in comparison with similar extracts of the pituitary gland of the horse and man very inactive in promoting ovarian growth in the immature rat, and experiments by Noble, Rowlands, Warwick, and Williams [1939] have shown histologically that they cause little follicle stimulation, but excessive luteinization in the ovaries of normal and hypophysectomized rats. Consequently, their antisera may be expected to possess, predominantly, an anti-luteinizing activity, which would cause a partial but differential neutralization of the luteinizing activity of the extract. The residual activity might, therefore, present a more efficient proportion of follicle stimulating to luteinizing action, for the stimulation of the ovaries of the intact immature rat. Maximum augmentation would indicate that the residual mixture contained an optimal balance of these two hormones.

It is likely, however, that this tentative explanation will have to be modified in view of new evidence now being obtained which suggests that the test animal's own pituitary gland is in some way involved in the augmentatory phenomenon.

SUMMARY

1. The serum of a dog injected daily for many weeks with an extract of ox pituitary gland augmented the gonadotrophic activity of extracts of ox and pig pituitary gland when tested on the ovaries of immature rats. The serum neither augments nor inhibits the thyrotrophic activity of the extract used for immunization. The absence of antithyrotrophic activity in this serum is probably explained in the light of the results of Rowlands and Young [1939].

2. The serum of a goat injected with an extract of pig pituitary gland containing thyrotrophic and gonadotrophic activity augmented the response produced by this extract in the ovaries of the immature rat. The pro-gonadotrophic activity of the serum was at a maximum after 6 weeks of injections, at a time when antithyrotrophic activity first appears. At 10 weeks the serum has a strong antithyrotrophic action, but a decreased augmentatory activity on the ovarian response.

3. An interpretation of the apparently augmentor action, as possibly due to selective depression of a normally predominant luteinizing action, needs review in relation to the possible action of the pituitary body of the test animal, which is under investigation.

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DIRECT GYNAECOGENIC AND INDIRECT OESTROGENIC ACTION OF TESTOSTERONE PROPIONATE IN FEMALE RATS

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THE numerous publications on the action of androgens on the female animal have shown that quite different types of effects may be produced by closely related substances of the male sex-hormone group. In some cases, however, the type of response has been found to vary with the substance under different experimental conditions. These findings would suggest that some of the effects ascribed to the various androgens may in reality be the resultant of a direct action and an indirect one through interaction with other hormones. In the following observations an attempt has been made to investigate fully the action of testosterone propionate in the female rat. The changes produced in ovariectomized adult rats have been compared with those after hypophysectomy, and the response of the immature hypophysectomized rat has been determined before and after ovarian stimulation by gonadotrophic hormones. As will be shown, testosterone propionate may under some conditions produce a characteristic direct effect, but its action may be modified by alteration of the experimental conditions.

The most consistent findings which have been reported to occur in female rats following testosterone propionate treatment may be summarized briefly.[1]

Vagina. Using immature intact female rats it has been found by numerous workers that testosterone produces premature opening of the vagina [Butenandt and Kudszus, 1935; Deanesly and Parkes, 1937; Rubinstein, Abarbanel, and Nader, 1938; Mazer and Mazer, 1939]. Smears from such animals were composed mainly of mucus, and histological examination of the vaginal epithelium showed typical mucification changes. Such changes were independent of ovarian influence since they were reproducible in immature animals after ovariectomy [Deanesly and Parkes, 1937; Rubinstein *et al.*, 1938].

Nathanson, Franseen, and Sweeney [1938] have confirmed that vaginal

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[1] The action of testosterone propionate on species other than the rat and the effects of other androgens are not within the scope of this paper, but are contained in reviews by Koch [1937], Korenchevsky [1939], and Varangot [1939].

opening occurs in intact or ovariectomized immature rats within 96 hours after a single injection of testosterone propionate, and similar effects were found in hypophysectomized and in hypophysectomized ovariectomized animals. In all cases the vaginal smear taken immediately after vaginal opening showed an oestrous response. On histological examination the vaginal epithelium was cornified. Salmon [1938a], however, found that in ovariectomized immature rats the vaginal smear showed an oestrous response for the first day after vaginal opening and thereafter a dioestrous condition. If the injections were continued the animal continued in oestrus for 5 to 8 days, but thereafter remained in dioestrus despite continued injections. Rubinstein et al. [1938] state that no oestrogenic activity was detected by smears on their animals injected daily. Fischer [1938] similarly did not find oestrogenic activity.

In the adult ovariectomized rat testosterone treatment is associated with a dioestrous mucous smear and mucification of the vaginal epithelium [Korenchevsky and Dennison, 1936; Nelson and Merckel, 1937; Korenchevsky, 1939]. In the intact normally cyclic female, the oestrous condition is inhibited and vaginal mucification is found. The oestrus-inhibiting action to testosterone is presumably due in part to the direct effect on the vaginal mucosa and partly to its antagonistic action against oestrogens [Korenchevsky, Dennison, and Brovsin, 1936; Korenchevsky, Dennison, and Hall, 1937; Browman, 1937; Wolfe and Hamilton, 1937; Hain, 1937].

In the hypophysectomized adult animal Parkes and Zuckerman [1938] have reported that testosterone propionate treatment was followed by vaginal oestrus and the vaginal epithelium showed cornified changes. As a possible explanation of this finding they suggest the lowered threshold of the hypophysectomized rat to oestrogen stimulation or the conversion of testosterone by the ovary into some oestrogenic compound.

Uterus. An increase in size of the uterus following testosterone has been noted by numerous workers. This has been reported in the immature animal and was independent of ovarian [Deanesly and Parkes, 1937; Nathanson et al., 1938; Salmon, 1938; Fischer, 1938] or pituitary action [Nathanson et al., 1938]. Similar hypertrophy was found in intact or ovariectomized adult rats [Korenchevsky, Dennison, and Brovsin, 1936; Nelson and Merckel, 1937]. The response of the uterus of the ovariectomized animal appears to be less than when the ovaries are intact, and secretory changes in the mucosa were found only when corpora lutea were present in the ovary [McKeown and Zuckerman, 1937; Nathanson et al., 1938]. Korenchevsky and Hall [1937] and Korenchevsky [1939] have described progestational lace-like folding of the uterine mucosa in both normal and ovariectomized rats. McKeown and Zuckerman [1937] found

in one of six rats treated with testosterone propionate that the uterus
responded to trauma by placentoma formation, while in others the
mucosa was of a progestational type. In ovariectomized animals, however,
no decidual or progestational changes were observed after similar treat-
ment or if animals were given preliminary oestrone injections. They
suggest that the decidual and progestational changes obtained were
dependent on the ovaries, presumably on the corpora lutea. Parkes and
Zuckerman [1938] failed to induce deciduomata in the uterus of hypo-
physectomized rats following testosterone propionate. Brooksby [1938]
was unable to produce decidual changes by trauma in normal or ovariec-
tomized rats following testosterone propionate. Astwood [1939] has
recently reported a similar failure. It would appear that testosterone pro-
pionate may induce progestational changes in the uterine mucosa of the
intact animal and decidual changes may occasionally be produced by
trauma. Decidual formation in ovariectomized rats has not been produced.
The observations recorded, therefore, indicate that the direct action on
the uterus of doses of testosterone propionate so far used is not equal to
that of progesterone. Under certain conditions, however, an indirect pro-
gesterone-like effect may be produced.

Ovaries. Testosterone propionate has under some conditions a gonado-
trophic action on the immature ovary. The formation of mature follicles
and the presence of corpora lutea have been noted in controlled experi-
ments [Hohlweg, 1937; Salmon, 1938 *a, b*; Nathanson *et al.*, 1938]. Rubin-
stein *et al.* [1938], however, state that in their animals, which received
1 mg. of testosterone propionate daily, follicular growth was definitely
inhibited. Fischer [1938] also found that follicular development was
retarded. Following hypophysectomy, testosterone did not have any
stimulating action on the immature ovary [Nathanson *et al.*, 1938]. Its
action, therefore, would seem to be an indirect one through the anterior
pituitary gonadotrophic hormones. It is possible that with large dosage
the pituitary function is suppressed whereas smaller doses cause stimula-
tion. The effects of prolonged injection, to be mentioned later, would
support such a contention.

In adult animals the corpora lutea of the ovary have been found to be
stimulated by testosterone treatment, especially when injections were
commenced when the animal was in oestrus [Wolfe and Hamilton, 1937;
Nelson and Merckel, 1937; McKeown and Zuckerman, 1937; Korenchevsky,
Dennison, and Hall, 1937].

Mammary glands. Testosterone has been shown to cause development
of the mammary gland in normal or ovariectomized rats [Selye, McEuen,
and Collip, 1936; Korenchevsky *et al.*, 1937; Nelson and Merckel, 1937].
This effect was dependent on the presence of anterior pituitary secretion,

since McEuen, Selye, and Collip [1937] could not detect any action in hypophysectomized rats. They also found (in male rats) that treatment of hypophysectomized animals with A.P.L., although stimulating the testes to produce male sex hormone, had no action on the mammary tissue comparable with that of testosterone in normal animals. An extract of the anterior pituitary, however, caused mammary hypertrophy.

Clitoris, preputial gland, female prostate. Testosterone stimulates the clitoris, preputial gland, and female prostate [Salmon, 1938 *a*; Korenchevsky, 1939]. Hall [1938] has described in detail the histological changes in the clitoris. The effect on these organs would appear to be due to direct stimulation.

Prolonged treatment. The effects of prolonged treatment with testosterone differ fundamentally from those previously described. Mazer and Mazer [1939] have shown that treatment for 102 days of immature rats with a dose of 1·5 mg. per week of testosterone propionate resulted in atrophic changes in the ovaries, with arrest of follicular and luteal development. The uterus, adrenals, and pituitary weights were also substantially below those of controls. Body-growth was unaltered. Adult animals similarly treated for 62–9 days showed atrophied ovaries, adrenals, and uteri. Such changes are probably resultant from an inhibitory effect on the anterior pituitary (comparable in some ways to prolonged oestrogen therapy), as Hamilton and Wolfe [1938] have demonstrated a lowered gonadotrophic hormone content in the pituitary after testosterone propionate treatment. Hertz and Meyer [1937] and Cutuly and Cutuly [1938] have shown in experiments using parabiotic rats that testosterone propionate suppresses the gonadotrophic activity of the pituitary.

In view of the lack of agreement on the effects of testosterone propionate on female rats, experiments have been conducted with special reference to the role played by the ovaries and pituitary.

<div align="center">METHODS</div>

Albino female rats of the Wistar strain, reared in this Institute, were used exclusively. The adult animals included those which had been subjected to bilateral ovariectomy, hypophysectomy, or both operations on the same animal. Immature animals (45 to 55 g.) were either intact or hypophysectomized. Synthetic testosterone propionate was dissolved in sesame oil at a concentration of 4·0 mg. per c.c. and 2 mg. were injected daily, except where indicated. Subcutaneous injections in most cases were started on the day following ovariectomy or hypophysectomy. In some instances various intervals were allowed to elapse before treatment was initiated. Synthetic progesterone was administered subcutaneously in a daily dose of 1 mg. dissolved in 0·5 c.c. of oil. When two different

preparations were given to the same animal they were injected on differen
sides. The gonadotrophic preparations used were PMS13, an extract o
the serum of pregnant mares [Leo], and UP10, an extract of human preg
nancy urine. Vaginal smears were made daily, and in immature animals
as soon as the vagina was found to be open.

The majority of animals were treated for 20 days and killed on the day
following the last injection. At autopsy the pituitary, ovaries, uterus and
vagina, adrenals, preputial glands, and first pair of thoracic mammary
glands, were dissected out and fixed in Bouin's solution. In some case:
the female prostate was also removed. The organs were weighed from
70% alcohol, and in most cases serially sectioned.

RESULTS

Adult Animals

(1) *Ovariectomized*. In four animals injections were commenced 10 days
after ovariectomy and the total dose of testosterone propionate adminis-
tered over the succeeding 28 days was 70 mg. The findings in these
animals were essentially similar to those in three others when injections
of 2 mg. daily were started on the day following ovariectomy, and con-
tinued for 20 days. During the injection period the vaginal smear showed
a dioestrous state and was composed chiefly of mucus. At autopsy the
uteri were markedly enlarged. They were not weighed because of the
variability of the portion removed during ovariectomy. The adrenal
weights were normal. Microscopic examination of the organs of these
animals, when compared with control ovariectomized rats, showed the
following features. The vaginal epithelium was thickened in numerous
folds and crypts, and the cells showed typical mucification, as may be seen
in Fig. 1. The uterus was hypertrophied and the mucosa heaped up into
projecting folds. The lining cells were elongated with basal nuclei and
occasionally contained large vacuoles. The mammary tissue was markedly
stimulated with proliferating ducts and well developed acini—Fig. 2.
The nipples were hypertrophied. The preputial glands were greatly
enlarged and the preputial groove was lined with thick stratified epithe-
lium which was heavily keratinized. In control animals the preputial
groove was less clearly defined and lined with only a few layers of small
round cells.

(2) *Ovariectomized and hypophysectomized*. Three animals had their
pituitary gland removed 9 days after ovariectomy, and were then injected
with 70 mg. of testosterone propionate over the succeeding 28 days,
similar to the four animals in (1). The changes found were similar to those
in (1) except for the mammary glands. Whereas the nipples showed
definite stimulation, the glandular tissue in these animals was undeveloped.
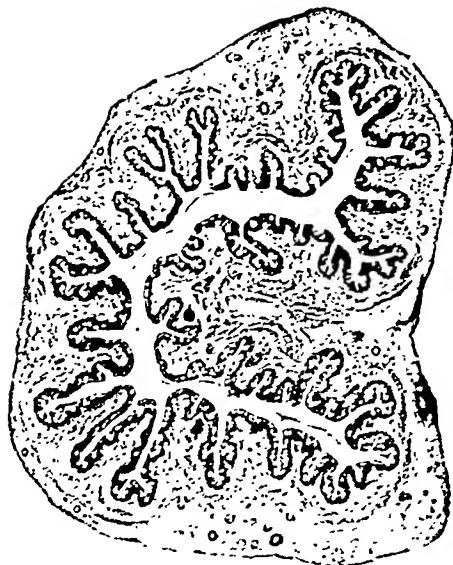
Fig. 1. Vaginal mucification after testosterone propionate in an adult ovariectomized rat. ×20
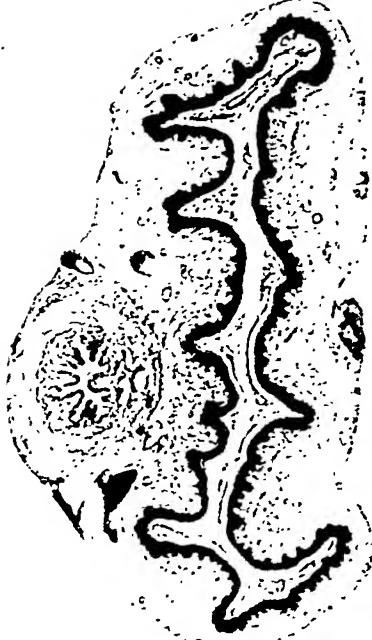
Fig. 4. Vaginal cornification after testosterone propionate in an adult hypophysectomized rat. × 18

Fig. 2. Mammary gland after testosterone propionate in an adult ovariectomized rat. × 10

Fig. 3. Mammary gland after testosterone propionate in an adult hypophysectomized rat. × 11

(3) *Hypophysectomized.* Two animals survived treatment with 100 mg. of testosterone propionate over 20 days, starting immediately after pituitary removal. A third animal was killed after receiving 40 mg. over 8 days. Four other animals were treated with 2 mg. daily; three of these were killed after 21 days (40 mg.) and one after 11 days (22 mg.). The vaginal smears on these animals were quite different from the animals in groups (1) and (2). An oestrous smear was obtained in all cases, and the vaginal epithelium was apparently maintained in an active condition, since the smears varied from oestrus to prooestrus throughout the 3 weeks. Control uninjected hypophysectomized animals in some cases remained in oestrus for some days after operation, but had always returned to dioestrus after 2 weeks. Histological examination of the vagina showed a thick epithelium either heavily stratified or stratified and cornified. In two animals mucification changes appeared to be superimposed in patches on stratified epithelium. The uteri were enlarged similar to those in (1) and (2), but the mucosa showed little or no folding. The lining cells were elongated and enlarged and contained occasional cyst-like spaces. The nipples were stimulated, but the mammary tissue was undeveloped— Fig. 3. The clitoris was similar to those in (1) and (2). The adrenal weights were within normal limits for hypophysectomized animals. The ovaries showed only small atrophic follicles, but well-maintained corpora lutea. Their weights were rather greater than those for untreated control animals.

Four animals were not injected until 10 days after hypophysectomy. Each received 40 mg. over 20 days. On the 3rd day after only two injections (4 mg.) a full oestrous vaginal smear was obtained in every case. This continued for 4, 6, 11, and 21 days respectively—the first animal returned to a dioestrous condition for the last 9 days before autopsy, but the other animals remained in prooestrus. The vaginal epithelium in the first rat was completely mucified, and in the last stratified with cornification—Fig. 4. In the other two animals areas of mucification and stratification were present. Other changes were similar to those in (3), except that the nipples appeared to be slightly less well developed. Corpora lutea were present in the ovaries, although the ovarian weights were less than those in (3). Two animals were allowed to remain for 3 weeks after hypophysectomy and were then injected with 8 mg. over 4 days. Both animals came into oestrus and showed stratification of vaginal epithelium.

Since rats which had been hypophysectomized for 10 days appeared to react well to testosterone injections, smaller doses were injected into four animals. Two received 0·4 mg. each over 3 days, and two 0·04 mg. each. One of the animals with the larger dose showed an oestrous smear, while both the animals with the smaller dose showed an oestrous reaction on at

least one day. At autopsy the vaginal epithelium was well developed and stratified in the animals which showed active smears.

The results of testosterone propionate treatment on adult rats have been summarized in Table I.

Table I. *Effects of testosterone propionate and progesterone on adult rats*

			Average per 100 g. body-weight				
No. of rats	Dose mg.	Days	Ovaries mg.	Adrenals mg.	Uterus mg.	Vagina	Mammary glands
Ovariectomized.							
2	0	21	—	18	—	A	—
4	71	28	—	15	—	M	+
3	40	21	—	16	—	M	+
Ovariectomized and hypophysectomized.							
1	0	28	—	9	—	A	—
3	71	28	—	5	—	M	—
Hypophysectomized.							
2	0	21	15	11	70	A	—
2	105	28	18	—	—	S	—
4	40	21	29	12	326	S	—
*4	40	21	17	10	435	S (+M)	—
†4	20	21	16	10	102	A	—

* Treatment started 10 days after hypophysectomy.
† Treated with progesterone alone.
M = mucified.   S = stratified.   A = atrophied.

In brief, it may be seen that stimulation of the vaginal epithelium with resulting stratification or cornification took place only in animals with intact ovaries. This response was still elicited 3 weeks after hypophysectomy. Stratified epithelium after prolonged treatment was gradually replaced by areas of mucification. Development of mammary glandular tissue was found only in animals with an intact pituitary gland, the ovaries having no role in such stimulation. Nipple growth occurred in all cases irrespective of the pituitary or ovaries. The clitoris and female prostate were stimulated in all cases, and the preputial groove was heavily keratinized. Folding of the uterine mucosa was less marked or absent in the hypophysectomized rats. The ovarian weights indicated that the corpora lutea were stimulated or maintained. These findings were very suggestive that the corpora lutea in the ovary were related to the production of vaginal oestrus by testosterone propionate.

Since it was possible that the secretion of progesterone by the corpora lutea might play some part in the results described, a control experiment was performed in which four adult animals received injections of 1·0 mg. of progesterone daily for 20 days, starting immediately after hypophysectomy. The vaginal smears in three of these animals showed a dioestrous condition, but in one case some activity was observed. On section the

vaginal epithelium was not stimulated (occasional cells distended with mucus were present) except in the case of the one rat. No effect on the mammary glands or nipples, uterus, adrenals, or preputial glands were found. The ovaries appeared similar to hypophysectomized controls. The partial activity in the one animal is difficult to explain, since hypophysectomy was apparently complete (although the sella turcica was not sectioned). The ovaries of this animal were the largest in the group, weighing 35 mg. These results have also been included in Table I.

It was thought that the immature rat would be of value in determining the role played by the corpora lutea in the response to testosterone propionate, since the effect on the immature ovary could be compared before and after development by gonadotrophic hormones. In the first instance the action of testosterone and progesterone on the ovary of the intact immature rat was obtained.

Intact Immature Rats

Testosterone propionate. Five rats were selected at weights from 32 to 37 g. so that daily injections of 2 mg. of testosterone propionate could be continued as long as possible before the animals reached maturity. They each received a total of 16 mg. and were killed on the 9th day, at an average weight of 59 g. (51–64 g.). A group of seven normal animals of average weight 60 g. (56–66g.) were killed as controls. Vaginal opening occurred in three of the injected rats on the 4th day of injection, in one on the 5th day, and in one on the 6th day. The vagina did not open in any of the control group. In one animal the first smear taken after the vaginal opening showed an oestrous condition; this on subsequent days remained as those from the other rats, in dioestrus. The vaginal epithelium at the end of the experiment showed typical mucification. The uteri were increased in weight and exhibited foldings of the mucosa. The ovaries averaged 10·8 mg. and those of the controls 15·7 mg. When sections of the ovaries were compared with those of the normal animals definite changes could be seen. In the control animals the largest follicles were only partly matured, but appeared fairly uniform in size, and contained well-developed antra. In the treated rats the ovaries contained 2 or 3 large mature follicles, whereas the remainder appeared to be generally less well developed than in the controls. Rupture of the follicle was not seen in any case and no evidence of luteinization could be found.

Five rats of 46–50 g. were treated by a single dose of 10 mg. of testosterone propionate in 1·0 c.c. of oil and killed 4 days later. Vaginal opening occurred in one rat on the day following injection, in one on the 2nd day, in one on the 3rd day, and in the remaining two on the 4th day. At autopsy the average body-weight was 58 g. (56–62 g.) and the average

weight of the ovaries was 11·6 mg. The histological picture of the ovaries and the uterus was essentially similar to that in the animals which had received daily injections.

Progesterone. A group of five rats of 32–9 g. in weight were injected with 1 mg. of progesterone daily for 8 days, and killed at an average of 62 g. (59–67 g.) on the 9th day. The vagina remained closed throughout in all animals. Although the ovarian weights were definitely less than the controls, the histological picture of both the ovaries and uterus was essentially similar to that in the control animals.

The effects of testosterone propionate and progesterone on intact immature rats are summarized in Table II.

Table II. *Effects of testosterone propionate and progesterone on normal immature rats*

No. of rats	Total dose mg.	Method	Body-weight g.	Average Ovaries mg.	Average Uterus mg.	Average Adrenals mg.
7	0	—	60·7	15·7	40·4	12·8
5	16	Daily testosterone	59	10·8	151	—
5	10	Single injection testosterone	58	11·6	128	12
5	8	Daily progesterone	62	11·3	45	—

These results confirm the observations that testosterone propionate produces stimulation of the ovarian follicles. Under the conditions described there was no evidence that the luteinizing hormone was stimulated. The actual weight of the ovary would appear to be definitely less after testosterone propionate or progesterone treatment. After testosterone propionate the uterus was hypertrophied and showed mucosal foldings. The adrenal weights appeared to be little affected.

Hypophysectomized Immature Rats

(a) *Testosterone propionate.* Five animals received 2 mg. daily, commencing immediately after hypophysectomy, for 20 days. Vaginal smears were made at the start of the experiment in only three animals. Vaginal opening occurred on the 5th day of treatment and in two cases an oestrous smear was obtained on this day. At the end of the experiment all five animals showed a dioestrous smear. Sections of the vagina showed typical mucification. The uteri were enlarged and showed foldings of the mucosa. The nipples were markedly stimulated, but the glandular tissue was undeveloped. The preputial glands were greatly enlarged and the preputial groove keratinized. The ovaries were identical with hypophy-

sectomized controls, and showed no evidence of maturation of follicles or luteinization.

(b) *Progesterone.* A group of four animals were injected with 1 mg. daily of progesterone for 20 days. Vaginal opening did not occur in any case. No changes were observed in sections of the vagina, ovaries, uterus, nipples or mammary glands, or preputial glands.

(c) *Testosterone Propionate and Progesterone.* A group of five animals were injected daily with 2 mg. of testosterone propionate and 1 mg. of progesterone for 20 days. Vaginal opening occurred on the 6th day of treatment and an initial oestrous smear was obtained in each case. Subsequent smears to the end of the experiment showed a dioestrous condition. Other changes were essentially similar to those after testosterone alone, although the uterine mucosa exhibited an increased degree of folding.

The results obtained in these three groups of animals are summarized in Table III.

Table III. *Effects of testosterone propionate and progesterone on hypo-physectomized immature rats*

| No. of rats | Total dose mg. | Method | Average | | | | | |
			Ovaries mg.	Uterus mg.	Adrenals mg.	Vagina	Mammary glands	Nipples
5	40	Testosterone daily × 20	4·7	210	7·4	M	—	+
5	20	Progesterone daily × 20	5·0	18	6·0	A	—	—
5	40	Testosterone daily × 20 and	5·7	240	7·2	M	—	+
	20	Progesterone daily × 20						

M = mucified.   A = atrophied.

From these results it may be seen that the action of testosterone propionate was not essentially altered when given simultaneously with progesterone in the doses used. An oestrous vaginal smear may be obtained immediately after vaginal opening occurs, but this was rapidly altered to a dioestrous condition which persisted to the end of the experiment. At autopsy the vaginal epithelium was heavily mucified. The folding of the uterine mucosa appeared to be increased by the supplementary progesterone treatment. Nipple growth was observed, but no development of mammary tissue occurred. Progesterone alone had no demonstrable effect.

(d) *Pregnant mare serum extract and testosterone propionate.* In the first experiment eight animals received a total of 3·0 mg. each of pregnant mare serum extract, PMS13, over 5 days, injections commencing the day following hypophysectomy. Five of these animals received 2 mg. of

testosterone propionate daily for 20 days, starting a day after the last injection of PMS13. Vaginal opening occurred on the 2nd or 3rd day of testosterone treatment and on the corresponding days in the three PMS-treated controls. No animal exhibited complete vaginal oestrus, although for the first week varying degrees of prooestrus were found. Histological changes in the treated animals were similar to those in (*a*). In the animals treated with PMS13 alone little difference could be seen from hypophysectomized uninjected controls.

From the weights and appearance of the ovaries of the above animals it was obvious that insufficient PMS extract had been given to produce corpora lutea. The experiment was repeated, therefore, using a larger dose of PMS13. Eight animals each received 15 mg. of PMS13 in a similar manner to the previous experiment, and five were injected with a similar dose of testosterone propionate. Vaginal opening occurred in all animals on the 4th day of treatment with PMS13, and the vaginal smear showed full oestrus. During the first week of treatment with testosterone the animals remained in oestrus or prooestrus. In one rat oestrus persisted to the end of the experiment, and in two others which survived the full period, prooestrus continued. In the two surviving animals treated with PMS13 alone, the vaginal smear showed a dioestrous condition for the last 13 days of the experiment. One animal survived only two injections of testosterone, and its ovaries weighed 96 mg. Another animal died after nine injections and showed an ovarian weight of 35 mg. One animal treated with PMS alone died on the day which corresponded to midway between the two previous animals with an ovarian weight of 62 mg. Examination of the rats which survived the full course of the experiment showed that in the testosterone-treated ones the vaginal epithelium was stratified and keratinized. The ovaries were composed almost entirely of large numbers of corpora lutea of all sizes. A few atrophic follicles were present. In animals which survived the longest the corpora lutea were discrete and surrounded by loose connective tissue. The uteri showed well-marked mucosal foldings, with hypertrophied cells, often vacuolated. Other changes were similar to those found in (*a*). In the animals treated with PMS13 alone the ovaries were indistinguishable from those after supplementary testosterone treatment. Despite the large size of the ovaries, the vagina and uterus had returned to an atrophic condition so that oestrogen secretion was apparently absent. Nipple or mammary stimulation was not present.

(*e*) *Human pregnancy urine extract and testosterone propionate.* Eight animals received a total of 5·0 mg. each of human pregnancy urine extract, UP10, over 5 days, injections commencing the day following hypophysectomy. Five of these received 2 mg. of testosterone propionate daily,

starting 1 day after the last injection of UP10. Only two rats survived 20 days of treatment, the other three being killed after 4, 8, and 10 injections. In the testosterone-injected group vaginal opening occurred in one rat on the 4th day and one on the 5th day of treatment. The initial vaginal smear showed an oestrous response in only one animal, and no subsequent activity was observed. In the three rats treated with UP10 alone vaginal opening did not occur. The histological changes in the testosterone-treated rats were similar to those described for group (*a*) except for the ovaries. These appeared to contain fewer immature follicles, and small bodies resembling corpora lutea were present. As many as three of these were found in one ovary and apparently represented the end-result of the diffuse luteinization which follows injection of such an extract. These were also present in the animals receiving UP10 alone, but were not so numerous or so large in size. Similar findings were present in the animals killed during the course of the experiment. The ovarian weight returned rapidly to normal, since 5 days after the last injection of UP10 the ovaries were approximately the same size as those found at the end of the experiment. This extract was tested on hypophysectomized rats by Noble, Rowlands, Warwick, and Williams [1939], and a 5 mg. dose over 5 days produced an average ovarian weight of 15 mg. on the day following the last injection, although the animals were not injected for 10–12 days after pituitary removal. The animals receiving UP10 alone showed changes similar to uninjected hypophysectomized controls except for the ovarian effects described.

The results of testosterone propionate treatment after PMS13 and UP10 are summarized in Table IV.

These findings show that when corpora lutea were developed in the immature ovary in response to pregnant mare serum they modified the mucification response to testosterone propionate, so that vaginal stratification and keratinization were produced. The luteinization caused by human pregnancy urine extract did not alter the response to testosterone, although small corpora lutea appeared to be present in the ovary at the end of the experiment. The ovaries after PMS13 treatment consisted of corpora lutea and a few atrophic follicles. Subsequent treatment with testosterone propionate did not maintain the corpora lutea and ovarian weight when compared with controls injected with PMS13 alone. Although the ovaries of the control PMS rats were of a large size, oestrogen secretion was absent, since the vagina and uterus were atrophied.

DISCUSSION

From the results described it is apparent that some of the effects of testosterone propionate observed in the female rat were related to the

presence of the ovaries and pituitary gland. To explain the mechanism by which these organs modify the effects produced is obviously impossible from the type of experiments described, but the findings may be considered along with those reported by other workers.

Testosterone propionate would appear to exert an indirect gonadotrophic action on the ovary through the anterior pituitary gland. Whereas the ovaries of hypophysectomized immature rats were unaffected, definite

Table IV. *Effects of testosterone propionate on hypophysectomized immature rats after preliminary gonadotrophic hormone treatment*

No. of rats	Treatment	Body wt. g.	Average Ovaries mg.	Average Uterus mg.	Average Adrenals mg.	Vagina	Mammary glands	Nipples
3	3 mg. PMS13.	61	4	26·6	4·6	A	—	—
5	3 mg. PMS13, 40 mg. testosterone propionate.	64	5·2	256·2	5·8	M	—	+
2	15 mg. PMS13.	46	37·5	20	3·0	A	—	—
3	15 mg. PMS13, 40 mg. testosterone propionate.	48	30·3	121·6	4·6	S	—	+
3	5 mg. UP10.	56	5·0	19·3	4·3	A	—	—
2	5 mg. UP10, 40 mg. testosterone propionate.	67	7·0	269	6·5	M	—	+

M = mucified.   S = stratified.   A = atrophied.

follicle formation was produced in intact animals. The follicles although mature had not ruptured and did not show any evidence of luteinization. The ovarian weights were less than in control animals, so that apparently the total secretion of pituitary gonadotrophin was less than normal. After prolonged treatment marked depression of pituitary secretion may occur, as shown by the atrophic condition of the ovaries [Mazer and Mazer, 1939], and by assay of the pituitary for gonadotrophic hormone [Hamilton and Wolfe, 1938]. The published data available gives little support to the contention of Freed, Greenhill, and Soskin [1938] that the different actions of testosterone propionate can be explained by a biphasic type of response of the pituitary, depending on the dosage of testosterone propionate employed. Many observers have noted that once corpora lutea are present in the ovary they are stimulated by testosterone. From the ovarian weights obtained in hypophysectomized adult rats it would appear that this effect is due to a direct action on the corpora lutea, and not neces-

sarily associated with liberation of pituitary luteinizing factor. When corpora lutea had been produced in immature hypophysectomized rats by preliminary treatment with pregnant mare serum extract testosterone propionate did not maintain the ovaries at their maximal size, but degeneration ensued similar to that in untreated controls.

The stimulating action of testosterone propionate on the mammary glands was only produced when the pituitary gland was intact and was independent of ovarian function, confirming the observations of McEuen *et al.* [1937]. It was readily seen, however, especially in the immature rats, that pronounced growth of the nipples occurred in the absence of the pituitary gland. Testosterone propionate, therefore, acts directly on the nipples, but has no effect on mammary glandular tissue unless the pituitary gland is present.

The initial production of vaginal oestrus in the immature rat, whether intact, ovariectomized, or hypophysectomized, seems to be rather curious, especially since most observers agree that the final result is typical vaginal mucification. In the experiments described an oestrous smear when obtained was always found on the day when the vagina was first open, and the rat never remained in oestrus longer than one day despite continued treatment. Other workers have reported, however, a positive oestrous smear for as long as 5 to 8 days [Salmon, 1938 *a*]. The significance of this initial, transitory, oestrous response is probably doubtful and may be related to the collection of epidermal debris which occurs, especially in view of the fact that the clitoris region undergoes extensive cornification. Such a source of contamination of the vagina has been discussed by Korenchevsky [1939]. The vaginal mucosa shows typical mucification after testosterone propionate treatment in the case of normal or ovariectomized adult rats, and in normal ovariectomized or hypophysectomized immature animals.

In hypophysectomized adult rats or in hypophysectomized immature animals after ovarian stimulation by pregnant mare serum, testosterone propionate produces stratification or cornification of the vaginal mucosa. In the hypophysectomized adult rat an oestrous response was obtained 3 weeks after pituitary removal. The corporea lutea of the ovary were apparently responsible for the oestrogenic action of testosterone propionate under such conditions. The ovarian follicles are unlikely to play any part in such a response, since they were found on histological examination to be atrophic, and also since the immature ovary after hypophysectomy is composed mainly of immature follicles, but vaginal mucification follows testosterone propionate treatment. The ability of the corpora lutea in the ovary of the hypophysectomized rat to modify the vaginal mucification produced by testosterone in ovariectomized animals

to vaginal stratification is curious, especially since vaginal mucification is produced in the intact adult rat. The experiments on immature hypophysectomized animals would indicate that an increased sensitivity to oestrogenic stimulation or a combined action with progesterone cannot explain the oestrogenic response. It is apparent that the corpora lutea when deprived of the effects of pituitary gonadotrophic hormones may respond in a different manner from those in normal rats. The type of luteinization following extracts of pregnancy urine in hypophysectomized immature rats was apparently different, either qualitatively or quantitatively, from that following extracts of pregnant mare serum, since with the former subsequent testosterone propionate treatment did not evoke a vaginal oestrous response.

Observations on the uterus, preputial glands, clitoris, and female prostate indicate that the stimulation recorded after testosterone propionate was through a direct action, as none of the operative procedures produced any alteration of the response.

Progesterone did not exert any action in the hypophysectomized immature rat. In the intact animal, however, the ovarian weights were considerably smaller than those of controls, suggesting that an inhibition of pituitary gonadotrophin secretion had occurred. A similar effect has been reported in adult animals by Selye, Browne, and Collip [1936]. In adult hypophysectomized animals progesterone treatment may be followed by the occurrence of occasional cells filled with mucus in the vaginal mucosa, as was also described by Selye, Browne, and Collip [1936]. Otherwise no consistent effects were observed; the oestrogenic activity in the one rat described was probably fortuitous. When testosterone propionate treatment was supplemented by progesterone in immature hypophysectomized rats no qualitative difference in the response was observed.

It is concluded from these experiments, therefore, that testosterone propionate produces, in the female rat, a direct stimulation of the clitoris, preputial glands, and female prostate (androgenic action); a direct stimulation of the uterus and nipples (gynaecogenic action). The direct effect on the vaginal mucosa (mucification) is modified after hypophysectomy by an additional indirect action leading to stratification with cornification of the vagina (indirect oestrogenic action) provided the ovaries are present and contain corpora lutea. Stimulation of the mammary glandular tissue (gynaecogenic action) is an indirect effect depending on the presence of the pituitary gland but not the ovaries.

SUMMARY

Testosterone propionate in a daily dose of 2 mg. for 3–4 weeks was found to produce stimulation of the mammary glands, a dioestrous condition

of the vaginal smears, and mucification of the vaginal epithelium in ovariectomized adult female rats. In hypophysectomized animals the mammary tissue was not developed, the vaginal smears showed an oestrous condition, and the vaginal epithelium was stratified or cornified. A vaginal oestrous response was still obtained when injections were commenced three weeks after removal of the pituitary gland.

Testosterone propionate injected daily or as a single dose was followed by stimulation of the ovarian follicles (without ovulation or luteinization) in normal immature female rats. The weight of the ovaries, however, was appreciably less than in control animals. In hypophysectomized immature rats testosterone propionate had no demonstrable effect on the ovary and produced typical vaginal mucification.

Treatment with testosterone propionate of immature hypophysectomized rats which had received preliminary ovarian stimulation by pregnant mare serum extract was followed by stratification of the vaginal mucosa. The ovaries regressed in size during treatment, similar to those in control animals which received pregnant mare serum extract alone. In these control animals the vaginal smear showed a dioestrous state during the last half of the experimental period and an atrophic condition on sectioning. Rats in a similar experiment, but in which ovarian stimulation was produced by preliminary treatment with pregnancy urine extract, failed to exhibit a vaginal oestrous response.

Vaginal opening occurred in all immature rats subjected to testosterone propionate treatment. An oestrous smear was obtained on the day of vaginal opening in some animals, whether intact or hypophysectomized. Following the initial oestrous smear, a dioestrous condition prevailed to the end of the experiment, although treatment was continued. In all cases, despite the various operative procedures, testosterone propionate produced stimulation of the nipples, preputial glands (with keratinization of the preputial groove), female prostate, and uterus.

Progesterone did not produce any consistent effect in hypophysectomized adult or immature rats, and its administration simultaneous with testosterone propionate did not modify qualitatively the effect of the latter in immature hypophysectomized animals. Progesterone injections were followed by a decrease in ovarian weights in intact immature rats.

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RECOVERY OF INJECTED OESTROGENIC SUBSTANCES FROM RABBIT URINE

By S. W. STROUD[1]

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EXPERIMENTS have shown that the amount of administered oestrogen that can be recovered from the urine of both human subjects and animals is generally very small. Luchsinger and Voss [1929], Kemp and Pedersen-Bjergaard [1933], Zondek [1934], Robson, MacGregor, Illingworth, and Steere [1934], and Mazer and Israel [1936], have performed experiments of this kind on human patients and have recovered only 3–12% of administered oestrone from the urine. Smith and Smith [1931] working with rabbits have shown that after the injection of 600–700 rat units of oestrone, 30 rat units or approximately 5% could be recovered from the urine, whilst Westerfield and Doisy [1937] have performed similar experiments with monkeys and have found that only 1·5–5·3% of injected oestrone could be recovered even from an ovariectomized-hysterectomized animal. Pincus and Zahl [1937], however, have studied the oestrogen metabolism in rabbits under varying conditions and in some cases claim recoveries up to 98% of the hormone injected, and Smith and Smith [1931] have shown that by simultaneous injection of progesterone with oestrone in rabbits the recovery of oestrone is raised to 80%.

In the majority of experiments, however, the amounts of the oestrogens administered were too small to permit of the isolation of the oestrogens in the urine, and it is possible that the oestrogenic activity found in the urine extracts, attributed to the original oestrogen injected, is due to a metabolic product which is itself oestrogenic. Again, the methods of extraction used by some workers are open to criticism. In some cases there is little doubt that insufficient care was taken to ensure complete liberation of the free oestrogens from their water-soluble conjugated forms by hydrolysis.

It was considered that it might be of great interest to investigate the fate of administered synthetic oestrogens in rabbits compared with that of oestrone, and the following experiments were carried out. The compounds used were 4 : 4′-dihydroxy–γ : δ–diphenyl-*n*–hexane [Campbell, Dodds, and Lawson, 1938], 4 : 4′-dihydroxy–α : β–diethylstilbene and 4 : 4′-dihydroxy–γ : δ–diphenyl–β : δ–hexadiene [Dodds, Golberg,

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In the majority of experiments, however, the amounts of the oestrogens administered were too small to permit of the isolation of the oestrogens in the urine, and it is possible that the oestrogenic activity found in the urine extracts, attributed to the original oestrogen injected, is due to a metabolic product which is itself oestrogenic. Again, the methods of extraction used by some workers are open to criticism. In some cases there is little doubt that insufficient care was taken to ensure complete liberation of the free oestrogens from their water-soluble conjugated forms by hydrolysis.

It was considered that it might be of great interest to investigate the fate of administered synthetic oestrogens in rabbits compared with that of oestrone, and the following experiments were carried out. The compounds used were 4 : 4'–dihydroxy–γ : δ–diphenyl–*n*–hexane [Campbell, Dodds, and Lawson, 1938], 4 : 4'–dihydroxy–α : β–diethylstilbene and 4 : 4'–dihydroxy–γ : δ–diphenyl–β : δ–hexadiene [Dodds, Golberg,

Lawson, and Robinson, 1938 *a*, *b*, and 1939]. In all cases where oestrogenic activity was found in the urine, oestrogens were isolated in quantities sufficient to account for the activity found.

Experimental

The technique for the administration of the compounds was standardized as follows:

The compounds were each dissolved in 80 ml. of sesame oil and two mature non-pregnant female rabbits were each injected with 2 ml. of this solution daily for a period of 20 days, the urine being collected over this period and also for a further 7 days after the last injection. The urine specimens were stored in a refrigerator after the addition of toluene as a preservative.

The method adopted for the extraction of the urines was as follows:

The urine was extracted with benzene in a continuous extractor for 20 hours. This extract containing the material extractable before hydrolysis was termed the 'free' extract. The extracted urine was then acidified with concentrated hydrochloric acid (50 ml. acid per litre of urine), boiled under reflux for 2 hours, and again extracted with benzene for 20 hours to give the 'combined' extract.

The benzene extract, 'free' or 'combined', was evaporated to a convenient volume, approximately 700 ml., extracted 3 times with 75 ml. portions of saturated sodium bicarbonate solution to remove acids, 5 times with 100 ml. portions of 2N sodium hydroxide solution to remove phenols, and finally washed twice with 50 ml. portions of water, the water washings being added to the sodium hydroxide washings. The sodium bicarbonate washings were extracted twice with two 75 ml. portions of ether, the ether solution being reserved. The sodium hydroxide washings were acidified to litmus with hydrochloric acid and extracted 4 times with 75 ml. portions of ether, the ether solution from the extraction of the sodium bicarbonate washings being used for the first two extractions. The ether solution was washed twice with 50 ml. portions of water, dried over sodium sulphate and evaporated to dryness, to give the phenolic fraction from the extracts.

For the purpose of biological assay each phenolic fraction was dissolved in acetone and made up to a definite volume. Aliquot portions of this solution were pipetted into test-tubes and sufficient acetone was added to make the volume up to 0·5 ml. 17·5 ml. of sesame oil were then added and the whole well shaken. For each test 5 ovariectomized female rats were used, each rat being injected twice daily with 0·5 ml. of the oil solution for 3 days, vaginal smears being taken on the 3rd, 4th, and 5th days. The unit adopted was under the lowest dose that would give 100% oestrous response under these conditions.

CONTROLS

Control experiments were previously carried out on the rabbits, which had been injected solely with sesame oil, and from the 'free' and 'combined' extracts made from the urine, phenolic fractions were separated as described. Large doses of these phenolic fractions when injected into ovariectomized rats failed to produce oestrous responses. Since very large doses of oestrogens were given to the rabbits in the later experiments, any small degree of activity in the control urine would of necessity be negligible.

4 : 4'-Dihydroxy-γ : δ-diphenyl-n-hexane

Two rabbits were injected with a total of 600 mg. over a period of 20 days.

Volume of urine	= 11,800 ml.
Weight of phenolic fraction obtained from 'free' extract	= 0·283 g.
Weight of phenolic fraction obtained from 'combined' extract	= 0·333 g.

The results are shown in Table I.

Table I

Amount injected	Recovery by biological assay			% recov. by biol. assay		
	Free	Combined	Total	Free	Comb.	Total
3,000,000 RU.	471,500 RU.	166,500 RU.	638,000 RU.	15·7%	5·6%	21·3%
= 600 mg.	≡ 94 mg.	≡ 33 mg.	≡ 127 mg.	—	—	—

The acetone solution of the 'free' phenolic fraction was evaporated to dryness and the residue dissolved in 20 ml. hot benzene. On cooling 78 mg. of needle-shaped crystals, m.p. 178° C., were obtained. On further concentration the benzene solution yielded another 14 mg. After recrystallization from benzene, crystals, m.p. 184° C., were obtained which did not depress the melting-point of 4 : 4'-dihydroxy-γ : δ-diphenyl-*n*-hexane (m.p. 184–5° C.). The acetone solution of the 'combined' phenolic fraction was treated in the same way as above. 26 mg. of crystals, m.p. 180° C., were obtained, which after recrystallization from benzene melted at 184° C. Again these crystals did not depress the melting-point of 4 : 4'-dihydroxy-γ : δ-diphenyl-*n*-hexane.

4 : 4'-Dihydroxy-γ : δ-diphenyl-β : δ-hexadiene

Two rabbits were injected with a total of 600 mg. over a period of 20 days.

Volume of urine	= 10,600 ml.
Weight of phenolic fraction from the 'free' extract	= 0·188 g.
Weight of phenolic fraction from the 'combined' extract	= 0·370 g.

The results are shown in Table II.

Table II

Amount injected	Recovery by biological assay			% rec. by biol. assay		
	Free	Combined	Total	Free	Comb.	Total
1,500,000 RU.	62,500 RU.	45,000 RU.	107,500 RU.	4·2%	3·0%	7·2%
600 mg.	25 mg.	∴ 18 mg.	∴ 43 mg.	—	—	—

The acetone solution of the 'free' phenolic fraction was evaporated to dryness and the residue dissolved in 10 ml. hot absolute alcohol. 10 ml. hot water were then added and the solution filtered hot and allowed to stand overnight in the refrigerator. 19 mg. of crystals, m.p. 226° C., were obtained which did not depress the melting-point of 4 : 4'-dihydroxy-γ : δ-diphenyl-β : δ-hexadiene, m.p. 227° C.

The acetone solution of the 'combined' phenolic fraction was treated in the same way as above. 13 mg. of crystals, m.p. 224° C., were obtained which did not depress the melting-point of 4 : 4'-dihydroxy-γ : δ-diphenyl-β : δ-hexadiene.

4 : 4'-Dihydroxy-α : β-diethylstilbene

Two experiments were carried out using this substance.

1. Two rabbits were injected with a total of 3 g. over a period of 20 days.

2. Two rabbits were injected with a total of 1·5 g. over a period of 20 days.

1. Volume of urine   = 10,640 ml.

     Weight of phenolic fraction obtained from 'free' extract   = 0·755 g.

     Weight of phenolic fraction obtained from 'combined' extract   = 1·377 g.

2. Volume of urine   = 11,100 ml.

     Only 3 l. of this urine extracted

     Weight of phenolic fraction obtained from 'free' extract   = 0·107 g.

     Weight of phenolic fraction obtained from 'combined' extract   = 0·154 g.

The results are shown in Table III.

Table III

Amount injected	Recovery by biological assay			% recov. by biol. assay		
	Free	Combined	Total	Free	Comb.	Total
8,570,000 RU.	1,217,000 RU.	940,000 RU.	2,157,000 RU.	14·2%	11·0%	25·2%
≡ 3·0 g.	≡ 0·426 g.	≡ 0·329 g.	≡ 0·755 g.	—	—	—
4,285,000 RU.	434,000 RU.	286,000 RU.	720,000 RU.	10·1%	6·7%	16·8%
≡ 1·5 g.	≡ 0·152 g.	≡ 0·100 g.	≡ 0·252 g.	—	—	—

1. The acetone solution of the 'free' phenolic fraction was evaporated to dryness, the residue then dissolved in 20 ml. boiling benzene, and the solution allowed to cool. 240 mg. of crystals, m.p. 161° C., were obtained. On concentration of the benzene solution and allowing to stand overnight another 174 mg. of these crystals were obtained. On recrystallization from benzene 326 mg. of crystals, m.p. 166° C., were obtained which did not depress the melting-point of 4 : 4'-dihydroxy-α : β-diethylstilbene, m.p. 168° C.

The acetone solution of the 'combined' phenolic fraction was treated as above. 259 mg. of crystals, m.p. 163° C., were obtained, which on recrystallization from benzene yielded 206 mg. crystals, m.p. 167° C., which did not depress the melting-point of 4 : 4'-dihydroxy-α : β-diethylstilbene, m.p. 168° C.

2. No chemical separation was carried out on the phenolic fractions.

Oestrone

Two rabbits were injected with a total of 1·5 g. over a period of 20 days.

Volume of urine	= 11,800 ml.
Weight of phenolic fraction from the 'free' extract	= 0·332 g.
Weight of phenolic fraction from the 'combined' extract	= 0·540 g.

The results are shown in Table IV.

Table IV

Amount injected	Recovery by biological assay			% recov. by biol. assay		
	Free	Combined	Total	Free	Comb.	Total
1,250,000 RU.	6,700 RU.	12,500 RU.	19,200 RU.	0·54%	1·0%	1·54%
= 1·5 g.	= 8 mg.	= 15 mg.	= 23 mg.	—	—	—

The acetone of the 'free' phenolic fraction was evaporated to dryness and the residue dissolved in 10 ml. hot glacial acetic acid and treated with Girard's reagent 'P' to obtain ketonic and non-ketonic fractions. The ketonic fraction was dissolved in a little hot ethyl acetate and the solution allowed to cool. 3 mg. of crystals, m.p. 246° C., were obtained. On recrystallization from ethyl acetate the crystals melted at 249° C. and gave no depression in melting-point when mixed with oestrone, m.p. 256° C.

The non-ketonic fraction was dissolved in a little hot ethyl acetate and allowed to cool. 16 mg. of crystals, m.p. 211° C., were obtained.

The acetone solution of the 'combined' phenolic fraction was treated with Girard's reagent in the same way as above. 5 mg. of crystals, m.p. 245° C., were obtained from the ketonic fraction. On recrystallization

from ethyl acetate these melted at 251° C. and gave no depression in melting-point when mixed with oestrone, m.p. 256° C.

The crystals obtained from the non-ketonic phenolic fractions from the 'free' and 'combined' extracts were mixed and recrystallized 3 times from ethyl acetate to a constant melting-point of 216° C.

The following figures were obtained from the analysis (Schoeller).

$$\text{Found C} = 78.9\% \qquad \text{H} = 8.95\%$$
$$C_{18}H_{24}O_2 \text{ requires C} = 79.3\% \qquad \text{H} = 8.9\%$$

When tested on ovariectomized rats the crystals were found to have a biological activity one-seventh that of oestrone.

DISCUSSION

In attempting to give significance to the excretion of these synthetic oestrogens it is necessary to regard with some reserve the relative amounts of the 'free' and 'combined' forms of these compounds found in the urine. Under the condition of collection and storage of the urine it is difficult to ensure that no changes due to putrefaction have occurred. It is possible that the combined forms of these synthetic substances are affected by putrefaction, as it has been shown [Callow, Callow, Emmens, and Stroud, 1939] that putrefaction of urine from human subjects liberates the naturally occuring hormones from their water-soluble conjugated forms, thus giving a high value for the free substances excreted. Allowing for this possibility, the amounts of the 'free' phenols found in the rabbit urine during the experiments are surprisingly large, when it is considered that with the natural hormones occurring in the urine of human subjects the amount of 'free' substances is so small, compared with the water-soluble combined forms, as to be negligible. It is also of interest to note the greater solubility of these synthetic oestrogens in urine compared with their solubilities in water; e.g. in the case of 4 : 4'-dihydroxy-α : β-diethylstilbene, 426 mg. of the 'free' phenol were found in 10·64 l. of urine, giving a solubility approximately 40 mg. per l., whereas in water the solubility is approximately 5 mg. per l.

In all cases the recovery of the synthetic oestrogens is greatly higher than that of the oestrone under the same conditions. 4 : 4'-dihydroxy-γ : δ-diphenyl-n-hexane and 4 : 4'-dihydroxy-α : β-diethylstilbene give recoveries of the order of 20%, compared with 7·2% for 4 : 4'-dihydroxy-γ : δ-diphenyl-β : δ-hexadiene and 1·5% for oestrone. These figures indicate the greater stability of the synthetic oestrogens in the body by comparison with oestrone and the possibility of a different metabolic path.

Special interest is attached to the oestrone experiment where, contrary to expectation, no oestriol was isolated from the urine. The non-ketonic

crystals, having an oestrogenic potency one-seventh that of oestrone when tested by our method, are doubtless a metabolic product of oestrone, and from consideration of the melting-point, analysis, and oestrogenic activity, probably β-oestradiol. The conversion of oestrone to β-oestradiol implies the reduction of the keto-group to the hydroxyl group, a process that is common in nature, e.g. the conversion of progesterone to pregnandiol, and testosterone to androsterone.

SUMMARY

1. A technique for the extraction of synthetic oestrogens from rabbits' urine has been described.

2. The recoveries of the synthetic oestrogens 4 : 4'-dihydroxy-γ : δ-diphenyl-n-hexane, 4 : 4'-dihydroxy-γ : δ-diphenyl-β : δ-hexadiene, and 4 : 4'-dihydroxy-α : β-diethylstilbene from rabbits' urine are compared with that of oestrone under the same conditions. The recoveries found were: for 4 : 4'-dihydroxy-γ : δ-diphenyl-n-hexane, 21·3%; for 4 : 4'-dihydroxy-γ : δ-diphenyl-β : δ-hexadiene, 7·2%; for 4 : 4'-dihydroxy-α : β diethylstilbene, 25·2% and 16·8%; and for oestrone, 1·5%.

3. A non-ketonic phenolic metabolic product of oestrone, probably β-oestradiol, has been isolated from the urine of rabbits receiving large injections of oestrone.

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MORPHOLOGICAL CHANGES IN FEMALE MICE RECEIVING LARGE DOSES OF TESTOSTERONE

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THE action of oestrogen overdosage has been studied by numerous investigators, and it was found that besides influencing the development of the sex organs, these substances exert a marked influence on the pituitary, the adrenals, the liver, &c. [Hohlweg, 1934; Wolfe, 1935 a, b; Selye, Collip, and Thomson, 1935; Selye, Harlow, and Collip, 1936; and Selye, 1938, 1939 a]. Since relatively few observations have been made up to the present along similar lines with chemically pure androgens, it appeared of interest to make a systematic study of the morphological changes produced by large doses of testosterone. As most of our previous work with the oestrogens was performed on female animals, we decided to use females for this work also, so as to make experimental conditions comparable.

The findings of previous investigators concerning the action of androgens on various organs are rather contradictory, partly because they used impure preparations, and partly because most of the authors who worked with chemically pure substances failed to realize that in order to obtain clear-cut overdosage symptoms, huge amounts of androgens must be administered since in most cases, weight for weight, the androgens are considerably less active than the oestrogens.

Concerning the action of the androgens on the *ovaries*, Castelnuovo [1937] and Regnier [1937, 1938] claimed to have obtained ovarian atrophy in fish treated with a testis preparation and testosterone propionate. In the toad (*Bufo arenarum*) Lopez [1938] noted no ovarian change following testosterone administration, while Shapiro [1936] and Shapiro and Zwarenstein [1937] obtained ovulation not only *in situ* but even in the excised ovary of *Xenopus laevis*. In the fowl, testis emulsions [Kobayashi, 1931] or daily administration of 5 capon units of 'proviron' (androsterone benzoate) [Schoeller and Gehrke, 1933] inhibit ovulation. 'Ludwig and v. Ries [1938] found that in a laying hen, 10 mg. of testosterone propionate administered on 2 successive days caused complete cessation of egg-laying during 1 month. In the guinea-pig, small doses of testis extract stimulate follicular growth while large doses cause ovarian atrophy and follicular atresia according to Migliavacca [1930 a, b, c]. On the other hand, Dempsey [1937] claimed that testosterone or androsterone benzoate in doses of

0·1 mg. daily does not increase follicular growth in this species, while Traina-Rao and de Palo [1938] claimed to have obtained follicular atresia and atretic luteinization in guinea-pigs treated with up to 60 mg. of testosterone propionate. In *Macacca mulatta* Zuckerman [1937] observed inhibition of follicular growth and luteinization following twice weekly administration of 25 mg. of testosterone propionate. Neumann [1932] induced sterility in mice treated with large doses of testicular tissue, while Parkes and Zuckerman [1938] claimed to have obtained luteinization of the ovaries in adult, but not in pubertal mice treated with testosterone propionate. From the short report of their work it is not quite clear how these authors were able to ascertain that the corpora lutea they observed were not merely those of the normal cycle but were produced by the treatment. Nor do they make any statement regarding the dose of testosterone propionate which they employed. Groher [1938] saw ovarian atrophy and follicular atresia in the ovaries of immature mice treated with a testicular extract. In the rabbit, Cotte, Martin, and Mankiewicz [1937] observed follicular atresia following testosterone propionate administration, while Gyarmati [1938] claimed that small doses inhibit follicular growth while large doses (up to 33 mg. within 3 weeks) result in corpus luteum formation. McEuen, Selye, and Collip [1937 a] administered 2 mg. of testosterone daily subcutaneously during 22 days to young female rats. They noted a marked inhibition of ovarian development, since the average weight of the ovaries in the treated group was 16 mg. as compared with 31 mg. in controls receiving 2 mg. of cholesterol daily. Histological studies of these ovaries have not been made. Wolfe and Hamilton [1937 a] administered 0·5 mg. of testosterone propionate to adult female rats daily for 10 days. They came to the conclusion that 'the ovaries of rats which received their injection either in estrus or metestrus contained corpora lutea which were definitely and consistently larger than those found in the ovaries of untreated rats killed during the normal estral cycle'. 'In the rats which received their first injection during diestrus, the results were more varied.' McKeown and Zuckerman [1937] claimed that adult rats receiving 1 mg. of testosterone daily responded with the formation of functional corpora lutea. The authors do not state, however, how they determined that the corpora they observed were actually produced as a result of the treatment. In immature female rats Hohlweg [1937] showed that androsterone fails to cause corpus luteum formation even if 50 mg. are given daily. Dehydro-androsterone, on the other hand, invariably produced precocious luteinization in doses of 2–3 mg. per day, while testosterone in doses of 5–10 mg. per day caused corpus luteum formation in some but not in all cases.

The action of androgens on the *adrenals* has been studied by Martin [1936], Poll [1933 a, b], and Starkey and Schmidt [1938], who found that the

physiological involution of the X-zone which usually occurs at puberty in the male mouse may be enhanced by the prepubertal administration of androgenic extracts. In the guinea-pig, Traina-Rao and de Palo [1938] claimed to have seen enlargement of the adrenal cortex after treatment with doses up to 60 mg. of testosterone propionate, while Bottomley and Folley [1938] found no change in the gross weight of the guinea-pig adrenal following treatment with numerous crystalline androgenic sterols. In the rat, McEuen *et al.* (1937 *a, b*) obtained a decrease in the size of the adrenals in females (in which the glands are normally larger than in males), while no such effect could be obtained in the male.

In the *hypophysis* of guinea-pigs, treated with the androgenic preparation 'enarmon', Hashimoto [1937] observed hyperplasia of the eosinophil cells but no change in the basophils. Franck [1937], on the other hand, states that 20–50 capon comb units of 'virex' administered within 2–5 days causes pituitary changes identical with those elicited by oestrogen treatment. Traina-Rao and de Palo [1938], who administered large doses of testosterone propionate to male and female guinea-pigs, also failed to see any difference between the pituitary changes caused by this substance and those elicited by oestrogens. Bottomley and Folley [1938] observed no significant changes in the weight of the hypophysis of young male guinea-pigs treated with numerous pure androgenic substances. In the rat, Bühler [1936] claimed to have obtained pituitary changes with the androgenic preparation 'proviron' (androsterone benzoate) which, though not as marked, were of the same nature as those produced by oestrogens. Wolfe and Hamilton [1937 *a, b, c*] noted that daily injection of 0·5 mg. of testosterone propionate during 10 days induced degranulation of the basophils but no noteworthy changes in the acidophils or chromophobes. Ten daily injections of 2 mg., on the other hand, caused degranulation of both types of chromophile cells and enlargement of the negative images of the Golgi apparatus. McEuen *et al.* [1937a] found that prolonged treatment with 0·2 mg. of testosterone daily has no effect on the gross size of the pituitary in the rat.

The *thyroids* of guinea-pigs treated with crude testicular extracts show signs of atrophy, according to Jedlowski [1935]. On the other hand, 5 mg. of testosterone propionate administered every 2nd day during a period of 8–20 days causes marked activation of the thyroid in young guinea-pigs, according to Carriére, Morel, and Gineste [1938]. Bottomley and Folley [1938], however, who used smaller doses of testosterone propionate and other androgenic sterols, were unable to observe any significant change in the weight of the thyroids in young male guinea-pigs.

The gross weight of the *liver* and *kidneys* is slightly subnormal in male castrate rats but returns towards normal following treatment with com-
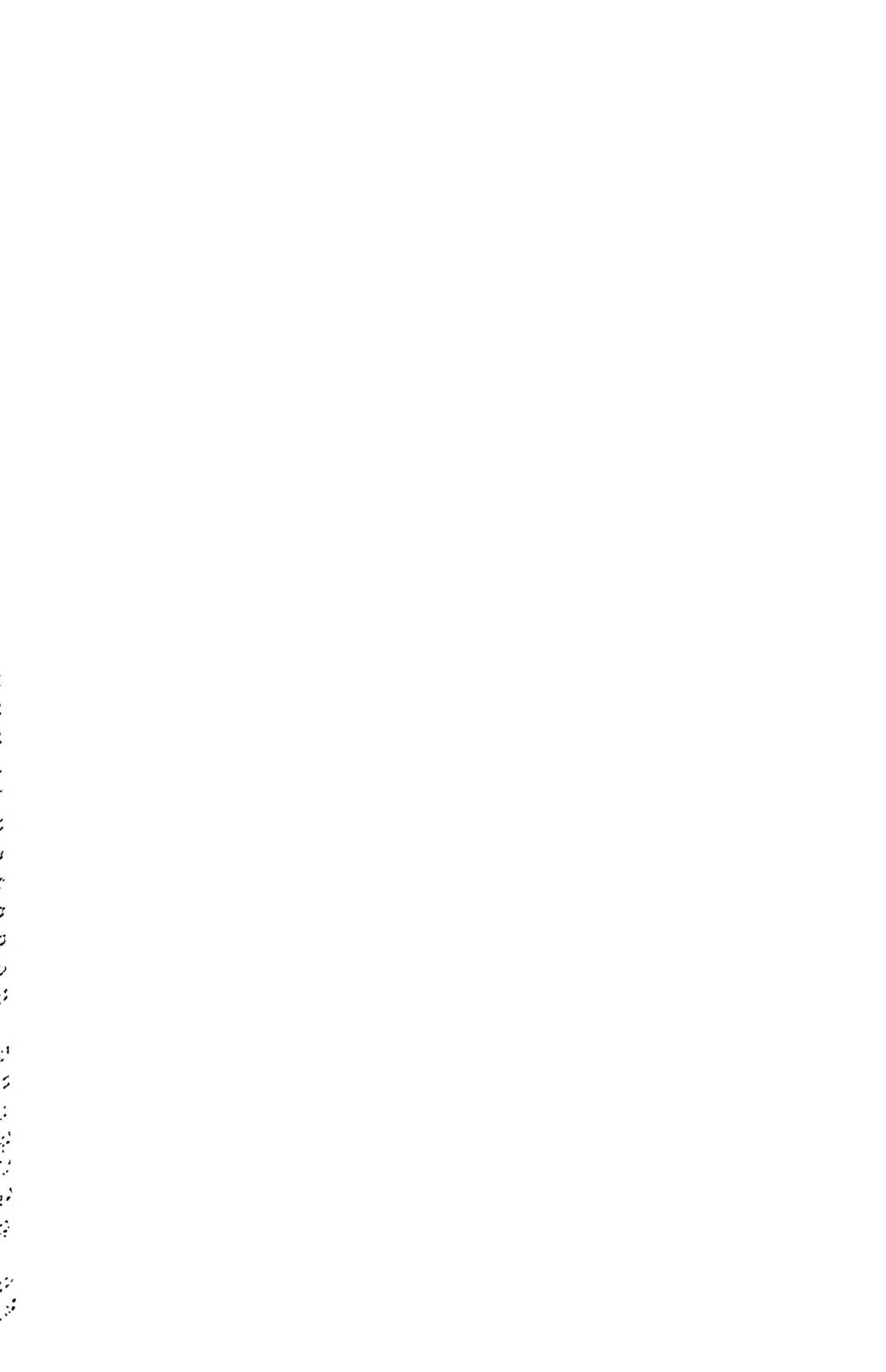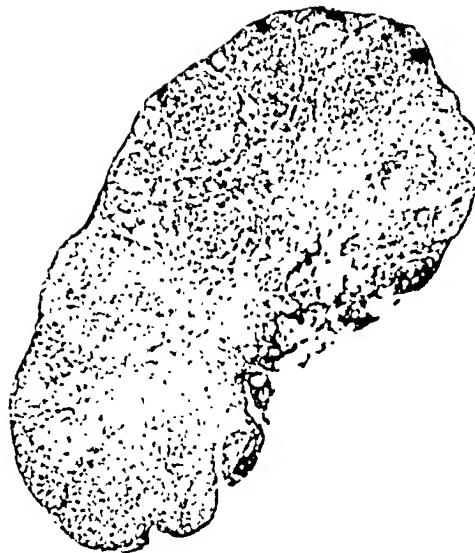
Fig. 1. Ovary of an untreated mouse showing numerous large corpora lutea and well-developed interstitial tissue. × 22
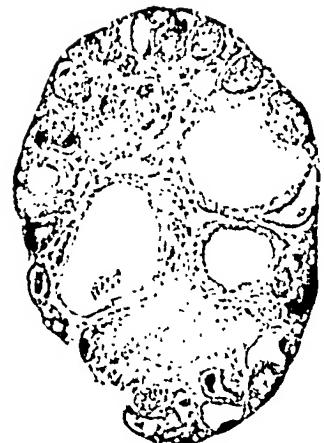
Fig. 2. Ovary of a testosterone-treated mouse. Note the large follicular cysts and the poor development of interstitial tissue. Active corpora lutea are absent but there are two scar-like remnants of involuted corpora lutea in this field. × 22
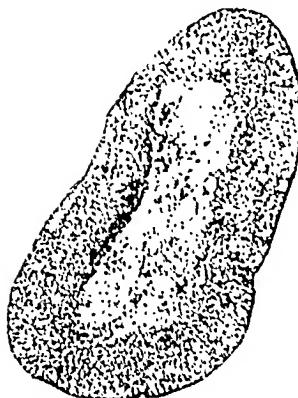
Fig. 3. General view of the adrenal of an untreated mouse. × 22
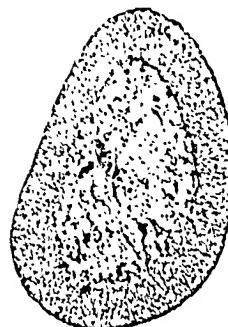
Fig. 4. General view of the adrenal of a mouse following testosterone treatment. Note the thin cortical layer and the well-developed medulla. × 22

mercial androgenic preparations, according to Korenchevsky and Dennison [1934].

<center>EXPERIMENTAL</center>

Seven adult grey female mice (Bar Harbor 'dba' strain) received 5 mg. of testosterone propionate in 0·2 ml. of Mazola oil daily subcutaneously during a period of 20 days. On the 21st day these animals and 6 untreated controls of approximately the same size and age were sacrificed and dissected. Their ovaries, adrenals, thyroids, hypophysis, liver, spleen, and kidneys were weighed after fixation in Heidenhain's 'Susa' mixture. We decided to weigh the organs after fixation because their dissection and accurate weighing is quite time-taking, and especially the small endocrine glands of mice tend to dry out so rapidly that they become useless for histological studies unless they are fixed immediately after death. The following table summarizes the organ weights in the testosterone-treated and normal animals.

Table I. *Organ weights of testosterone-treated mice. All organ weights are expressed in mg. and the body-weight in g.*

Testosterone-treated mice

	1	2	3	4	5	6	7	Average
Body-weight	23	24	20	21	21	23	24	22
Ovaries	7·2	6·9	5·6	7·3	6·0	8·0	9·0	7·1
Adrenals	6·4	6·0	6·0	5·6	6·1	7·0	5·8	6·1
Thyroids	3·8	4·0	3·6	3·4	4·0	3·7	3·8	3·8
Hypophysis	2·2	2·3	2·0	2·0	2·1	2·1	1·6	2·0
Kidneys	568	700	558	610	552	636	644	609·7
Liver	1570	1880	1370	1490	1500	1670	1770	1607·1
Spleen	168	190	128	146	150	153	156	155·9

Untreated mice

	1	2	3	4	5	6	7	Average
Body-weight	21	26	26	25	26	30	35	25·5
Ovaries	18·0	16·0	20·0	22·0	15·0	17·0	21·0	18·4
Adrenals	10·0	8·0	11·0	9·2	10·0	8·0	8·1	9·2
Thyroids	6·0	4·0	4·8	3·8	5·1	5·2	4·1	4·7
Hypophysis	3·8	2·4	2·9	3·0	3·1	3·2	3·1	3·1
Kidneys	380	484	530	406	490	486	532	472·6
Liver	1500	1300	1500	2000	2040	2070	1700	1730·0
Spleen	123	110	70	138	155	120	140	122·3

All organs were examined histologically, 3–5μ sections being made after embedding in paraffin. All sections were stained with haematoxylin eosin and with iron haematoxylin except the pituitaries, which were stained with the method described by Selye and McKeown [1935], which proved particularly useful for the study of the various anterior lobe cells.

The *ovaries* (see Figs. 1 and 2) of the testosterone-treated animals were much smaller than those of the controls. Histological examination showed that compact corpora lutea were invariably absent and

most of the ovarian tissue consisted of large follicles, follicular cysts, and a few cystic corpora lutea, having an unusually large central cavity filled with colloid. In two cases large 'blood-points' were observed, that is to say, greatly enlarged follicles whose antrum was filled with blood while the granulosa cells were perhaps in the process of beginning luteinization. Like the cystic follicles and cystic corpora lutea, these blood-points were unusually large because of their wide central cavity. The interstitial tissue of the ovary was atrophic and the stroma of the gland consisted almost exclusively of ordinary connective tissue. This is in marked contrast with the condition of the normal control ovaries which in the case of 'dba' mice are extremely rich in interstitial cells. The excessive development of the interstitial tissue in this strain of mice accounts at least partly for the fact that their ovaries are much larger than those of most other strains we examined.

The fact that testosterone given in large doses stimulates follicle maturation stands in marked contrast with the results obtained following the administration of oestrogens in massive doses. The latter, as is generally known [Hohlweg, 1934; Selye *et al.* 1935; &c.], cause luteinization of the ovary. In the case of the oestrogenic substances we were able to prove that this effect is due to stimulation of the pituitary, because in hypophysectomized animals the oestrogens fail to produce new corpora lutea [Selye and Collip, 1936]. It is possible that oestrogens stimulate the pituitary to release luteinizing hormone, while testosterone causes the gland to discharge the follicle-stimulating principle.

Since the gross size of the ovaries decreases after testosterone administration, it is easy to understand that those authors who limited their observations to the determination of the ovarian weights concluded that testosterone causes atrophy of the female gonad. It is now known that androgens may exert a progesterone-like effect on the endometrium. Hence one may have to revise the conclusion of those investigators who claim to have obtained functional corpora lutea with testosterone because in their treated animals uterine trauma resulted in deciduoma formation. It may be that the deciduomata which occurred in the testosterone-treated animals were due to the testosterone itself and not to corpus luteum hormone secreted by corpora lutea which were believed to have been produced by the androgen. The fact that testosterone tends to stimulate follicular growth may also explain why animals receiving gonadotrophic substances simultaneously with testosterone respond predominantly with follicle maturation [Selye, 1939*b*].

The *adrenals* (Figs. 3–6) of our testosterone-treated mice were considerably smaller in weight than those of the control group. This is by no means purely due to involution of the X-zone, since this zone is not
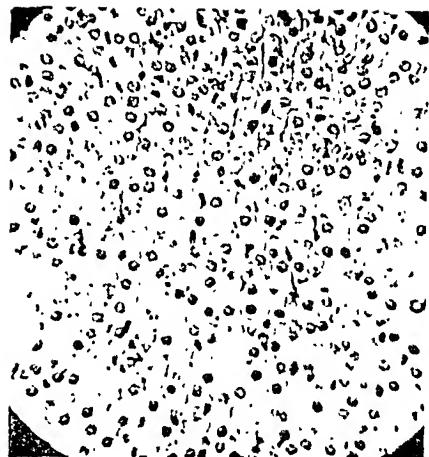
Fig. 5. Portion of the adrenal cortex of an untreated mouse. × 250
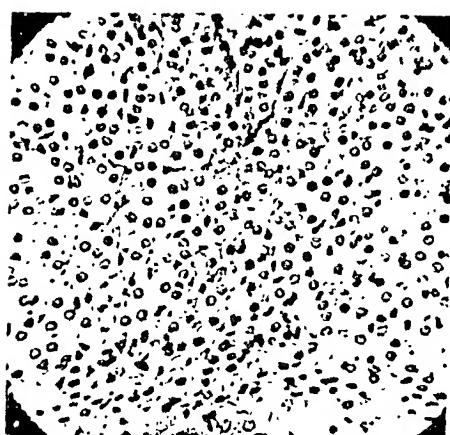
Fig. 6. Portion of the adrenal cortex of a testosterone-treated mouse. Note the poor development of the cytoplasm and the decrease in the total size of these cells. × 250
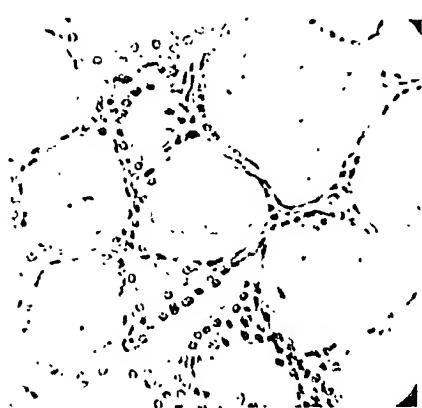
Fig. 7. Thyroid of an untreated mouse. × 250
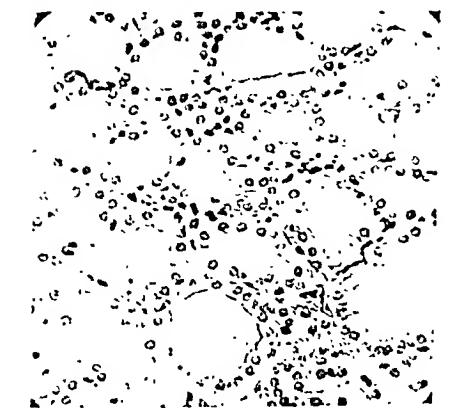
Fig. 8. Thyroid of a testosterone-treated mouse. Note enlargement of the follicular cells and absorption vacuoles in the peripheral part of the follicular colloid. × 250

well developed under any conditions in either sex in this strain. Histological studies of the adrenals revealed that the atrophy was mainly, if not entirely, due to involution of the cortical cells, but all three zones participated. Figs. 5 and 6 show that there was an actual decrease in the cytoplasm content of the individual cortical cells as compared with the normal. In view of the fact that large doses of oestrogen increase the size of the adrenal cortex, it appears that with regard to their action on the suprarenal gland, testosterone and the oestrogens also exert contrary effects.

The *thyroids* (Figs. 7 and 8) of the testosterone-treated mice are larger than those of the controls, and histologically, in most cases, there appeared to be some increase in the height of the follicular epithelium and some colloid absorption under the influence of the androgen. This, however, was not always as obvious as in the case shown in our photographs.

The *hypophysis* was significantly smaller than normal following testosterone propionate treatment. This is another example showing the contrary effect of testosterone and the oestrogens, since it is well known that the latter elicit marked enlargement of the hypophysis, sometimes resulting in tumour formation. It should be emphasized particularly that we found no evidence of degranulation in the chromophile cells of the anterior lobe in these mice, so that our histological observations confirm the contention that contrary to what has been claimed by several other investigators whose names were mentioned in the introductory section of this communication, even the large doses of testosterone used in the present experimental series failed to elicit any pituitary change comparable to that produced by the oestrogens.

The *liver* and *spleen* of our treated animals showed no significant histological changes. The gross weight of the liver was likewise approximately normal, and although the average weight of the spleen was slightly increased in comparison with the control group, the individual variations in the weight of this organ were so pronounced that it is doubtful whether treatment had any effect on the splenic tissue. We wish to emphasize that we have never seen liver necrosis and periportal hepatitis in these testosterone-propionate-treated mice, although such changes were extremely frequent and often resulted in death in mice receiving large doses of various oestrogens [Selye, 1939a].

The *kidneys*, as will be seen from the table, are greatly enlarged as a result of testosterone propionate administration. Since this effect of testosterone was the object of a special study and has been dealt with in detail in a separate communication [Selye, 1939c], it will suffice to mention here that the enlargement is mainly due to hypertrophy of the cells of the

proximal and distal convoluted tubules and of the parietal lamina of Bowman's capsules while the glomeruli and the collecting tubes are apparently intact.

SUMMARY

Daily administration of 5 mg. of testosterone propionate to adult female mice during a period of 20 days causes a decrease in the total size of the ovaries, disappearance of normal corpora lutea, and marked follicle stimulation with the formation of follicular and occasionally of corpus luteum cysts. The adrenal cortex becomes atrophic, the hypophysis decreases in weight without, however, showing any indication of a degranulation of the anterior lobe chromophile cells. The kidneys are markedly changed, and histological examination reveals hypertrophy of the proximal and distal convoluted tubules and of the parietal lamina of Bowman's capsules. The liver and spleen show no significant morphological alteration. Even such high doses of testosterone propionate as have been used in these experiments do not produce toxic actions comparable to those observed following oestrogen overdosage.

With respect to the changes produced in the ovaries, the adrenals, and the pituitary, the effect of testosterone propionate appears to be the opposite of that of the oestrogens.

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EFFECTS OF SYNTHETIC OESTROGENS AND CARCINOGENS WHEN ADMINISTERED TO RATS BY SUBCUTANEOUS IMPLANTATION OF CRYSTALS OR TABLETS

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In a preliminary report [Noble, 1938 *a*] it was shown that the subcutaneous implantation of crystals of synthetic oestrogenic substances was followed by an inhibition of body-growth for a prolonged period in adult rats, a decreased rate of growth in young animals, and a loss of gonadotrophic hormone from the anterior pituitary gland associated with atrophy of the reproductive organs. These changes could be produced by a substance such as diethylstilboestrol, the molecular structure of which had but little resemblance to the naturally occurring oestrogens. Up to the present time no qualitative differences in the effects of oestrogens on the various parts of the body have been demonstrated. Since a large series of synthetic oestrogenic substances which had been prepared in this laboratory were available for experimental study, it has been possible to compare the effects of representative active substances from different chemical groups with closely related substances of little or no oestrogenic activity. The various oestrogenic substances which have been used in these experiments have been previously described by Cook, Dodds, Hewett, and Lawson [1934]; Cook, Dodds, and Lawson [1936]; Dodds, Golberg, Lawson, and Robinson [1938 *a*, 1938 *b*]; Dodds and Lawson [1938]; Dodds, Lawson, and Noble [1938].

The observations of Haddow, Scott, and Scott [1937] that some hydrocarbons which had carcinogenic but not oestrogenic properties produced a reduction in body-growth and a decreased sexual activity in rats appeared of interest in their relationship to the problem under investigation. If the action of these non-oestrogenic hydrocarbons on growth and the pituitary gland was similar to that of oestrogenic substances, it would demonstrate that the molecular structure necessary for the production of oestrus was not essential for the production of the other effects. Experiments have been conducted, therefore, with various carcinogenic substances.

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METHODS

White rats of the Wistar strain which were bred in this Institute have been used throughout. They were raised and maintained solely on a diet of compressed food of the same composition and form as described by Thomson [1936]. Lettuce was given as a supplement once a week. Special precautions were taken to ensure that a constant supply of water was always available.

In the preliminary experiments 10 or 100 mg. of crystals of the substances to be investigated were implanted directly into the subcutaneous tissues of immature or adult rats. With such a method of administration the dosage could only be approximately estimated, since it was impossible in most cases to weigh the crystals remaining at the end of the experiment. In some cases tablets of 10 mg. size were made by pressure, without excipient or binding agent, and implanted subcutaneously, as described by Deanesly and Parkes [1937, 1938]. Synthetic substances were found to be less suitable for making into tablets than was oestrone, and considerable variation in hardness must have occurred. When removed from the animal the connective tissue covering was carefully dissected off and the remains of the tablet dried and weighed.

When the rats were treated with gonadotrophic extracts of pregnancy urine the injections were given in equal doses over 5 days, and the animal killed on the following day. The animals were weighed at regular intervals during the experiment, and the body-weight has been used as an index of body-growth. At autopsy the various organs were dissected, fixed in Bouin's solution, and then weighed, or weighed from 70% alcohol, as indicated. In some cases the pituitary gland was weighed in the fresh state.

For ease in presentation, the various substances tested have been designated by a number, and are given in Table I. The approximate oestrogenic activity has been calculated from previously published tests and expressed in International Units per gram. This activity had been determined in ovariectomized rats by the vaginal smear method following six subcutaneous injections, over 3 days, of the substance dissolved in sesame oil, or in the case of Sub.26 and Sub.27, in aqueous alcohol solution.

RESULTS

Crystal implantation in mature rats. The substances employed were implanted into groups of three or five male and female mature rats, and the animals killed 4 weeks after crystal implantation. In Table II and Table III the changes in body-weight and endocrine weights are recorded.

The results in general were similar to those previously reported [Noble,

1938*a*] in that substances which had strong oestrogenic properties exerted an inhibiting effect on body-growth and on anterior pituitary function. In male rats the gain in weight which was exhibited after the administration of inactive substances was greater than that shown by the females. The male rats, however, were used at a lower initial weight, and exhibit a more rapid growth curve than the female at this size. The former having

Table I

Sub.1. 1 : 2-Dihydroxy-1 : 2-diphenyl-acenaphthene
Sub.2. Triphenyl carbinol
Sub.3. 4-Hydroxy-dibenzyl
Sub.4. Dibenzyl
Sub.5. 1-Keto-1 : 2 : 3 : 4 : 5 : 6 : 7 : 8-octahydroanthracene
Sub.6. 9 : 10-Dihydroxy-9 : 10-di-*cyclo*hexyl-9 : 10-dihydro-1 : 2 : 5 : 6-dibenzanthracene
Sub.7. Phenanthrene
Sub.8. Chrysene
Sub.9. 1 : 2-*cyclo*pentenophenanthrene
Sub.10. 1 : 9-dimethyl-phenanthrene
Sub.11. 1 : 2 : 5 : 6-Dibenzanthracene
Sub.12. 1 : 2-Benzpyrene
Sub.13. Methylcholanthrene
Sub.14. 4 : 4′-Dihydroxy-α : β-dibenzylidene-dibenzyl
Sub.15. β : β-Di(4-hydroxyphenyl)-propane
Sub.16. 4 : 4′-Dihydroxydiphenyl-methane
Sub.17. Diphenyl-α-naphthyl carbinol
Sub.18. Stilbene
Sub.19. 4 : 4′-Dihydroxy-stilbene
Sub.20. 4-Hydroxy-stilbene
Sub.21. 1 : 2-Dihydroxy-1 : 2-di-α-naphthyl-acenaphthene
Sub.22. Triphenyl-ethylene
Sub.23. 9 : 10-Dihydroxy-9 : 10-di-*cyclo*pentyl-9 : 10-dihydro-1 : 2 : 5 : 6-dibenzanthracene
Sub.24. 9 : 10-Dihydroxy-9 : 10-di-*n*-propyl-9 : 10-dihydro-1 : 2 : 5 : 6-dibenzanthracene
Sub.25. 4 : 4′-Dihydroxy-α : β-dimethyl stilbene
Sub.26. 4 : 4′-Dihydroxy-β : δ-diphenyl-β : δ-hexadiene
Sub.27. 4 : 4′-Dihydroxy-α : β-diethylstilbene

a more constant and uniform growth are the more reliable test animals on which to determine growth inhibition. Inspection of the male animals showed that the testes were retracted and that the scrotal tissue had practically disappeared. Marked atrophy of the testes was associated with cessation of androgen secretion, since the prostate and seminal vesicles were reduced to a minimum size. In the female, atrophy of the ovaries was to be expected from the inhibition of the pituitary gonado-trophic hormone secretion, but, since oestrone may exert a direct effect on the corpora lutea of the rat [Hohlweg, 1934; Nelson, 1934; Selye, Collip, and Thompson, 1935; Wolfe, 1935; Ellison and Burch, 1936], and as Robson has shown [1937] even after hypophysectomy in the rabbit, the final results were rather varied. The weight of the uterus also showed a considerable variation. Enlargement of the adrenals and the pituitary gland was noted in both sexes.

Some degree of differentiation in the various effects was exhibited in
the case of a few substances. The ovarian weights recorded after the
administration of the highly active Sub.26 were almost twice as great as
those after treatment with the equally active Sub.27. It appeared from

Table II. *Effect of synthetic oestrogens and carcinogens on adult male rats—
Crystals implanted for 4 weeks*

Sub- stance	I.U. per g.	Rat dose mg.	No. rats	Av. body-weight		Av. per 100 g. body-weight*				
				Initial g.	Change g.	Adre- nals mg.	Semi- nal ves. mg.	Pros- tate mg.	Testes mg.	Pitui- tary mg.
Sub.1	0	100	3	142	+48	17	260	255	1205	3·8
„ 2	0	100	3	149	+60	12	233	314	1180	3·5
„ 3	0	100	3	143	+58	14	293	317	1247	3·5
„ 4	0	100	3	152	+80	14	273	239	1118	3·7
„ 5	0	100	3	148	+65	14	271	250	1172	3·4
„ 6	0	10	3	125	+84	13	201	226	1268	3·7
„ 7	0	10	5	159	+75	13	315	338	1136	3·6
„ 8	0	10	5	163	+53	14	302	282	1276	3·9
„ 9	0	10	4	168	+75	11	232	228	1048	3·4
„ 10	0	10	5	140	+61	14	325	318	1143	3·8
„ 11	(carcino- genic) 0	10	5	147	+75	13	278	287	1212	3·6
„ 12	(carcino- genic) 0	10	5	130	+82	13	256	284	1173	3·7
„ 13	(carcino- genic) 0	10	5	145	+45	15	273	279	1278	3·8
„ 14	200	10	3	129	+55	16	308	294	1441	4·3
„ 15	120	100	3	150	+52	19	188	236	1024	4·0
„ 16	120	100	3	113	+79	16	163	210	1272	4·2
„ 17	120	100	3	137	+70	16	219	264	1206	3·9
„ 18	480	100	3	141	+49	20	164	204	1085	4·3
„ 19	2400	100	3	123	+57	17	120	180	1268	4·0
„ 20	2400	100	3	137	+41	21	112	152	1015	4·3
„ 21	2400	100	3	135	+53	18	142	184	1395	4·2
„ 22	2400	100	3	133	+ 6	30	23	59	644	5·3
„ 23	12,000	10	3	125	+84	13	201	226	1268	3·7
„ 24	24×10^4	10	3	123	+30	32	30	63	731	5·1
„ 25	60×10^4	10	3	150	+42	19	191	206	1189	4·3
„ 26	20×10^6	10	3	137	− 7	41	26	57	393	11·3
„ 27	24×10^6	10	3	140	+42	19	146	176	969	4·1

* Pituitary gland weighed in fresh state—other organs after fixation.

inspection and microscopic appearance that the corpora lutea were large
and well maintained. Sub.26, therefore, appears more efficient in stimu-
lating the corpora lutea than any other substance tested. In the animals
treated with Sub.22 the well-marked atrophic changes due to inhibition
of pituitary secretion were present, but the size of the pituitary gland was
normal. This was in striking contrast to the weight of the pituitary gland
recorded after treatment with Sub.26 and Sub.27. In addition, Sub.22
was found to exert a much greater general action than would be expected

from its relatively low oestrogenic property. This does not appear to be readily explained, unless the effects on the pituitary gland of this substance are substantially greater than its action on the vagina. The opposite finding, that some substances such as Sub.23 exerted a lesser effect than would be expected from the oestrogenic activity, may readily be

Table III. *Effect of synthetic oestrogens on adult female rats—Crystals implanted for 4 weeks*

Sub-stance	I.U. per g.	Rat dose mg.	No. rats	Av. body-weight		Av. per 100 g. body-weight*			
				Initial g.	Change g.	Adrenals mg.	Uterus mg.	Ovaries mg.	Pituitary mg.
Sub.1	0	100	3	154	+18	27	262	31	6·8
„ 2	0	100	3	157	+19	26	286	32	5·6
„ 3	0	100	3	168	+ 9	31	349	33	7·1
„ 4	0	100	3	155	+19	26	297	34	5·6
„ 5	0	100	3	161	+ 8	29	244	38	6·0
„ 6	0	100	3	160	+14	27	231	35	5·2
„ 14	200	10	3	150	+13	24	493	27	5·7
„ 15	120	100	3	168	+14	27	306	34	6·0
„ 16	120	100	3	156	+17	26	192	30	5·5
„ 17	120	100	3	136	+22	26	418	36	6·0
„ 18	480	100	3	152	+ 4	29	326	36	6·2
„ 19	2400	100	3	163	− 5	25	258	27	6·2
„ 20	2400	100	3	163	+ 7	30	209	36	6·2
„ 21	2400	100	3	162	+12	27	256	29	5·2
„ 22	2400	100	3	148	−22	47	442	26	7·2
„ 23	12,000	10	3	141	+21	29	403	36	5·3
„ 24	24×10⁴	10	3	157	+ 5	33	249	26	5·4
„ 25	60×10⁴	10	3	149	+15	35	279	29	6·9
„ 26	20×10⁶	10	3	163	−10	49	1179	48	16·5
„ 27	24×10⁶	10	3	127	+30	42	288	25	9·2

* Pituitary gland weighed in fresh state—other organs after fixation.

explained by variations in solubility. In cases where the solubility was extremely low it was to be expected that even active substances would produce little effect when administered in the form of crystals. A substance which was readily soluble was Sub.27. In this instance the effects of 10 mg. of crystals were apparent only from 14 to 20 days. While the differential actions exhibited by some substances appeared quite definite, the variation in solubility makes it impossible to state whether these were of a qualitative rather than a quantitative nature. The three carcinogenic hydrocarbons which were tested did not produce any appreciable change in body-growth or in the weights of the gonads. Since these substances have a low solubility, it is possible that the amount absorbed was not great enough to produce any effect.

Crystal implantation in immature rats. Only the three oestrogenic substances which exerted the greatest inhibiting action on growth and on the secretion of the pituitary gonadotrophic hormones in the adult rats

were used for implantation into immature animals. The effect on growth in these animals was in striking contrast to the inhibition which occurred in animals over 130 g. in weight. As was previously shown [Noble, 1938 a], Sub.26, when implanted into adult animals, suppressed growth. When given to rats at an average of 65 gm. in weight a slight increase in growth took place. When the animals were started at 36 g., however, only a slight alteration from the normal growth occurred. It appeared, therefore, that in immature rats the pituitary gland might secrete such large amounts of growth hormone that the oestrogenic substance was not capable of entirely suppressing its effect. On the other hand, some extra-pituitary growth mechanism might be present in young animals, which was uninfluenced by oestrogenic substances. This problem will be discussed later, but the growth of the immature rats following crystal implantation has been included in Table IV. In some cases a diminution was observed.

Following crystal implantation in immature rats it was found that, although growth continued, inhibition of pituitary gonadotrophic secretion was complete in that the gonads remained in the infantile state. On gross and microscopic examination the gonads resembled those of an animal following hypophysectomy. The functional capacity of the pituitary to secrete gonadotrophic hormone in such animals was determined by the response of the ovary to injections of pregnancy urine extract. Treatment with such extracts produces well-developed corpora lutea with an increased ovarian weight in immature intact rats, but only a diffuse luteinization of the ovary in hypophysectomized rats. This difference in response is related to the stimulation of the anterior pituitary gland to secrete gonadotrophic hormone [Noble, Rowlands, Warwick, and Williams, 1939]. The effects of pregnancy urine extract on immature female rats 3 weeks after crystal implantation are shown in Table IV. The ovaries of these animals were increased in weight and showed corpora lutea. The response, therefore, was similar to that observed for intact immature rats. In some cases ovarian weights greater than those produced in normal rats were found. Although the secretion of pituitary gonadotrophic hormone was inhibited by the oestrogenic substance, the condition of the gland was such that it could respond normally to pregnancy urine extract.

Tablet implantation in immature rats. In order to examine the effect of the more active oestrogenic substances over a prolonged period they were made into 10 mg. tablets which were implanted subcutaneously into immature rats. The animals were killed from 10 to 24 weeks after the tablets were inserted. Control operations were performed on some animals, which were kept under similar conditions for comparative study. A 10 mg. tablet of Sub.27 was implanted into each of five male and five female rats. Ten weeks later two of the male and three of the female animals were

killed so that the approximate size of the gonads could be ascertained (Table V). At 11 weeks the tablets were removed from two of the remaining males and the two females. The growth of the ten rats for the first 10 weeks may be compared with that of normal rats in Fig. 1. The subse-

Table IV. *Effect of implanted oestrogenic crystals and injections of pregnancy urine extract on immature female rats*

No. of rats	Sub-stance no.	Rat dose mg.	Dura-tion Days	Av. body-weight		Av. per 100 g. body-weight*			
				Initial g.	Change g.	Adre-nals mg.	Uterus mg.	Ovaries mg.	Pitui-tary mg.
8	Normal controls	21		39	+57	—	—	—	—
5	Normal controls	21		40	+50	—	—	—	—
5	27	10	23	41	+52	29	157	16	9·0
5	26	10	28	66	+23	46	386	20	13·7
	Treated with pregnancy urine extract 2·0 mg. UP10								
5	controls	—	—	94	—		176	81	—
5	26	10	26	37	+41	—	166	60	—
5	22	100	26	36	+52	—	127	101	—
5	22	100	21	58	+28	—	133	72	—
	Treated with pregnancy urine extract 100 m.u. Physex								
5	controls	—	—	51	—	—	164	53	—
5	27	10	24	39	+42	28	323	123	9·6

* Pituitary gland weighed in fresh state—other organs after fixation.

Table V. *Effect of 10 mg. tablets of Sub.27 on immature rats*

Rat no.	Dura-tion Weeks	Final wt. of tablet mg.	Av. body-weight		Av. per 100 g. body-weight*				
			Initial g.	Change g.	Adre-nals mg.	Seminal vesicles mg.	Pros-tate mg.	Testes mg.	Pitui-tary mg.
748	10	1	42	+101	24	28	20	108	5·5
752	10	2	46	+84	29	80	40	146	10·0
						Uterus mg.	Ovaries mg.		
759	10	5	41	+76	36	293	6	—	18·8
760	10	3	38	+69	31	290	4	—	25·2
763	10	3	38	+79	35	280	5	—	12·8

* Pituitary gland weighed in fresh state—other organs from 70% alcohol.

quent growth of the animals which had the tablets removed is also included.

The four animals from which the tablets were removed were allowed 16 weeks in which to return to normal, and they were then mated to normal animals. Both the treated male rats sired litters, while one of the females gave birth to and raised a normal litter. Apparently the reproductive organs had sufficiently recovered, from the atrophic condition seen in the rats listed in Table V, for normal reproduction to take place.

The effects of 10 mg. tablets of Sub.26 were determined similarly in

four male rats and nine females over a period of 17–18 weeks. Fig. 2 shows the growth curve of these rats. Four of the female animals were injected with pregnancy urine extract for the 5 days before they were killed, and the results are included with those obtained in the remaining rats in Table VI.
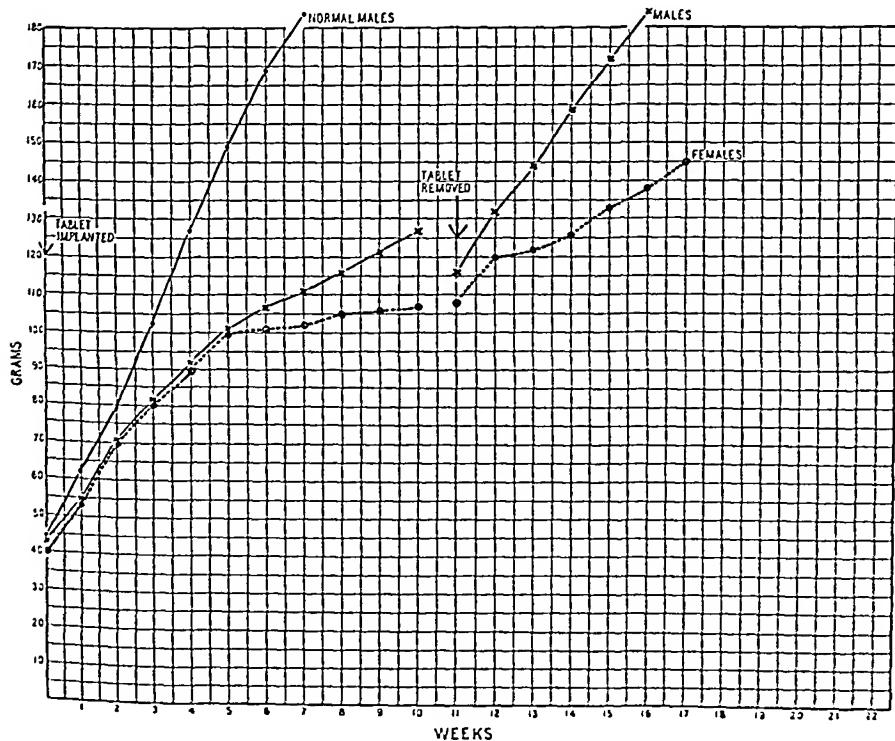
FIG. 1. Effect of 10 mg. tablets of Sub.27 on body-growth.

The growth curve of the rats in these experiments showed the striking difference in response which was found between the immature and mature rats. Growth was slightly inhibited initially but the effect became marked at about 100 g. body-weight, when growth was extremely small. The individual variation in body-weight during the course of the experiment represented by Fig. 2 was small when compared to normal rats. As may be calculated from Table VI, the weights of male animals at autopsy varied from 121 to 149 g. and the female weights from 107 to 122 g. The animals from which the tablets were removed resumed normal growth. The gonadotrophic activity of the pituitary gland was suppressed throughout the experiment. The response to pregnancy urine extract differed from that obtained in rats after a short period of crystal implantation.

Instead of normal corpora lutea being formed only diffuse luteinization of the ovary was produced. Such an effect was similar to that described for hypophysectomized rats. In three animals the small follicles were almost completely replaced by luteinized tissue, but in one rat follicular development with typical corpora lutea was found.

Table VI. *Effect of 10 mg. tablets of Sub.26 on immature rats*

Rat no.	Dura- tion Weeks	Final wt. of tablet mg.	Av. body-weight Initial g.	Av. body-weight Change g.	Adre- nals mg.	Seminal vesicles mg.	Pros- tate mg.	Testes mg.	Pitui- tary mg.
749	20	control	46	+323	8	240	285	820	2·4
750	20	,,	46	+260	13	239	258	946	2·6
751	20	,,	40	+322	9	336	327	763	2·7
754	20	,,	37	+296	12	300	279	948	1·8
757	20	,,	48	+274	10	247	171	782	2·4
798	18	3	50	+71	24	91	51	128	9·0
799	18	4	52	+81	21	101	37	97	14·2
802	18	1·5	42	+106	33	75	38	291	6·7
820	18	1·0	47	+102	20	57	20	161	7·0

Rat no.	Dura- tion Weeks	Final wt. of tablet mg.	Av. body-weight Initial g.	Av. body-weight Change g.	Adre- nals mg.		Uterus mg.	Ovaries mg.	Pitui- tary mg.
761	20	control	39	+138	24	—	231	32	5·0
765	20	,,	43	+174	20	—	354	21	4·1
766	20	,,	41	+129	25	—	241	31	5·8
767	20	,,	42	+158	21	—	267	35	4·5
788	17	2·0	54	+63	27	—	Pyometria	5	14·5
789	17	1·5	44	+73	31	—	376	5	7·6
790	17	3·0	49	+73	27	—	362	10	10·6
791	17	4·0	42	+72	50	—	316	7	8·7
792	17	<1·0	43	+64	37	—	387	8	14·0
†793	17	1·0	41	+77	27	—	286	Fibrosis	14·4
†795	17	3·0	46	+75	28	—	292	25	17·3
‡796	17	1·0	40	+68	34	—	370	31	11·1
‡797	17	4·0	40	+74	34	—	Pyometria	17	7·8

* Pituitary gland weighed in fresh state—other organs from 70% alcohol.
† Each received total 2 mg. UP10.
‡ Each received total 4 mg. UP10.

DISCUSSION

Body-growth. That the injection of naturally occurring oestrogens would modify body-growth in the rat has frequently been recorded [Spencer, Gustavson, and D'Amour, 1931, 1932 *a*; Halpern and D'Amour, 1934, 1936; Zondek, 1936].[1] With adequate dosage of synthetic oestrogenic substances it has been shown that inhibition of growth, as measured by body-weight, was readily produced in animals over 120 g. As far as could be determined no substance without oestrogenic properties produced this effect. The most probable mode of action of oestrogenic substances

[1] Deanesly [1939] has recently described in detail the changes following tablet implants of oestrone and oestradiol in male rats.

in suppressing growth in adult animals is by an inhibition of growth hormone of the anterior pituitary gland. The lowered gonadotrophic hormone content of the pituitary gland and the observations of Spencer *et al.* [1932 *b*] that growth hormone would maintain normal growth in oestrone-treated rats would support such a suggestion. The action of
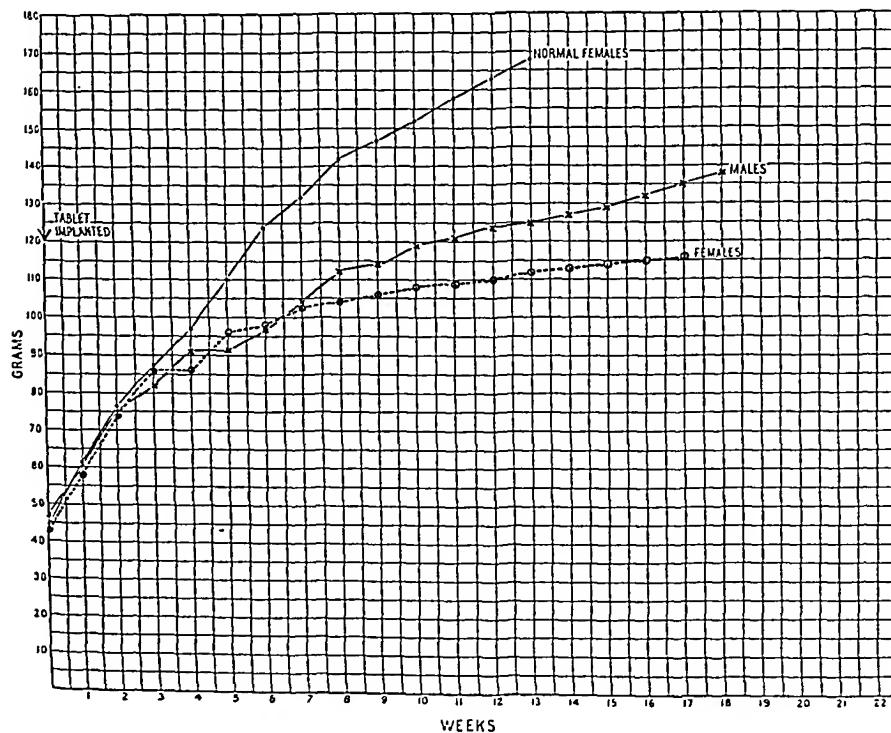
FIG. 2. Effect of 10 mg. tablets of Sub.26 on body-growth.

oestrogens on young animals appeared of considerable interest in that only slight retardation of growth occurred. It would seem improbable that inadequate dosage was entirely responsible for the inability to arrest growth in these animals. The experimental evidence, therefore, is very suggestive that growth of the young animal is affected by an extra-pituitary factor. In further experiments to investigate this possibility it has been found impossible to stop body-growth, although a slower growth-rate than reported above has been successfully produced by the continuous oral administration of oestrogenic substances in aqueous solution. From the experiments described, however, it is impossible to state whether the growth of the immature rat depends on an extra-pituitary factor or not, and if so, whether the slight effect of oestrogens

on this is due to inhibition of associated and additional pituitary-controlled growth or direct growth inhibition.

Gonadotrophic activity. The atrophic changes in the gonads which follow the repeated injection of oestrogens have been frequently noted. That these were associated with a lowered gonadotrophic hormone content of the pituitary were reported by Meyer, Leonard, Hisaw, and Martin [1930]; Bialet-Laprida [1933]; Lipschutz [1935], and Noble [1938 a]. Treatment of such animals with pituitary gland implants or gonadotrophic extracts produced typical stimulation of the gonads [Moore and Price, 1930, 1932; Diaz, Phelps, Ellison, and Burch, 1938; Noble, 1938 b]. The active oestrogenic substances described have been found to produce, through inhibition of the anterior pituitary gland, an atrophy of the gonads in adult animals, or to arrest their development in young animals. Male and female animals which were treated by tablet implantation over a period of 10 weeks were able to sire or produce normal litters following removal of the tablets.

Pregnancy urine extract has been shown to cause stimulation of the ovaries and corpora lutea formation when administered to animals 3 weeks after crystal implantation. Spencer *et al.* [1932 b] have previously reported a similar observation in rats injected with oestrone. After a longer period of oestrogen treatment pregnancy urine extract caused in three out of four rats only diffuse luteinization of the ovary, resembling the typical effect of such extracts on hypophysectomized animals. A diminished response to pregnancy urine extract has been noted by Fevold, Hisaw, and Greep [1935] after prolonged oestrone administration. Selye *et al.* [1935] have pointed out that ovaries depressed by oestrone treatment do not respond normally to A.P.L. administration, but when corpora lutea are produced by A.P.L. they may be enlarged by concurrent and subsequent oestrone treatment. In the animals described, which were treated for short periods with oestrogens, the pituitary gland, although not secreting gonadotrophic hormones, must have been in such a condition that it could be stimulated by pregnancy urine extract. Once corpora lutea were produced they would be stimulated directly by the oestrogen, resulting in an increased size of the ovary. Prolonged treatment by oestrogen tablets apparently affected the pituitary so that it was not stimulated by pregnancy urine extract.

Microscopic examination of the ovaries and testes of the rats after treatment showed a picture which was identical with that following hypophysectomy. There was no evidence that secretion of any part of the gonadotrophic complex of the anterior pituitary gland had continued.

Other effects. Enlargement of the pituitary gland occurred and the largest observed was 25·2 mg. per 100 g. body-weight. Severinghaus [1937] has suggested from histological evidence that the anterior pituitary cells

appear to be hyperactive after oestrone treatment. In the experiments described there was no evidence to suggest that hypersecretion of any pituitary hormone had taken place, unless it was of the adrenotrophic hormone. The detailed histology of the pituitary gland of the animals in these observations will be reported with Mr. C. L. Foster.

Adrenal enlargement following oestrogen therapy has been produced in rats by Selye *et al.* [1935] and Ellison and Burch [1936]. This effect was apparently dependent on pituitary secretion, since it did not occur after hypophysectomy. Selye [1937] has shown that adrenal hypertrophy may be produced in the 'alarm reaction' by various noxious stimuli, and Selye, Harlow, and Collip [1936] have described the alarm reaction produced by oestrogens. The increase in adrenal weight which has been shown to follow the administration of synthetic oestrogens would appear to be specifically related to oestrogenic activity, since inactive substances were without effect. It has been found that adrenal hypertrophy is not produced by synthetic oestrogens in rats which have been hypophysectomized.

Although the testes of immature rats remained in the infantile condition after tablet implantation, the seminal vesicles in some cases appeared to be disproportionately large. Microscopic examination of these showed that they were devoid of any secretion and composed almost entirely of solid fibrous tissue. Weights up to 101 mg. per 100 g. body-weight were recorded. This stimulation of fibrous tissue is similar to that produced by oestrone, as described by Freud [1933].

Pathological changes. The general toxicity of the substances used in these experiments was slight. The animals remained in apparent good health, and except for a certain amount of alopecia, appeared normal. Sections of the liver failed to show any evidence of damage, although mild transitory jaundice has been noted in two rats after treatment with active substances. After prolonged treatment the mammary glands occasionally showed cyst-like spaces filled with a milky fluid. There was no evidence of excessive epithelial hyperplasia. Pyometria was frequently observed in female animals with uterine hypertrophy. In such cases inflammatory changes and fibrosis sometimes extended to the ovaries, which occasionally showed the presence of small cysts.

Absorption. The rate of absorption of Sub.22, Sub.26, and Sub.27 were calculated from the alteration which took place in the weight of the tablet after implantation. When crystals were used it was possible to obtain only an approximate idea of the time taken for them to disappear. As would be expected, crystals were dissolved much more rapidly than tablets. In the case of Sub.27 this was very marked, since the effects of 10 mg. of crystals lasted only 2–3 weeks, and after 4 weeks no crystals could be found at the site of implantation. A tablet of similar weight,

OBSERVATIONS RELATED TO THE SWELLING
OF SEXUAL SKIN IN RHESUS MONKEYS

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KROHN and Zuckerman [1937], Fisher and Zuckerman [1937], and Ayk-royd and Zuckerman [1938] have shown that the process of swelling of the sexual skin of immature female monkeys (*M. nemestrina* and *M. mulatta*) induced by oestrogenic stimulation is associated with the following occur-rences: a general water-retention; increase in the size of the cells of the con-nective tissue of the sexual skin; the presence of a viscous exudate easily drained from cut surfaces of this tissue; variations of the water content of the tissue between cells and exudate; the exudate having a variable but high total osmotic pressure, which may exceed that of serum, and a total protein content of *ca.* 2·5 %.

A further investigation of the exudate was undertaken in the hope that information might be obtained on the mechanism of sexual-skin swelling.

MATERIALS

Immature female rhesus monkeys (*Macaca mulatta*) were injected with 100 μg. oestrone in oil daily for varying periods. Blood, sexual skin, and other tissues were removed rapidly under ether anaesthesia.

Serum was obtained from blood withdrawn from the heart, or in larger amounts by rapid carotid bleeding. After a clot had formed and had contracted (3–4 hours), the serum was poured off and finally cleared by centrifuging; little haemolysis occurred. The composition of the serum was not affected by variation of the method of obtaining the blood.

Exudate was obtained by squeezing chopped swollen skin gently through muslin. The exudate was centrifuged to separate fat from suspen-sion, and used within a few hours of separation.

Table I illustrates the approximate state of sexual-skin swelling of the monkeys used; as far as possible they are arranged in order of advance-ment in the stages of swelling, as judged by inspection. The observations made in each case are shown in Table I.

We are greatly indebted to Professor H. W. Florey for samples of duo-denal mucins.

Table I

Number of Monkey	Days of oestrone dosage	State of swelling					Exudate	Chemical analysis	Ultra-centrifuge	Swelling in vitro	Measurement of fibroblasts
		Coming up			Summit	Adolescent type					
		Anus	Vulval lips	Pubic lobe							
421	5	+++	+++	++			−			+	+
405	6	++	++	+			+		+		
407	9	++	++	+			+	+	+		
425	10	+	++++	+			−			+	+
402	9	+	++	++			+	÷	+		
408	13	+	++	++			+		+		
411	14	+	++	++			−			+	
228	12	+		+++++			+		+		
229	9				+++++		+		+		
410	23				++++	+	+	+	+	−	+
401	26				++++	+	+	+	+	−	
403	18				+	++++	+	+	+		
406	31					+++++	÷	+		−	+
409	44					Very late	+−			−	+

METHODS AND RESULTS

Analysis of Exudate and Serum

The exudate examined was presumably extracellular, obtainable by gentle squeezing of the chopped tissue. None could be obtained by this means from tissue in the earliest stages of swelling nor from that in which subsidence of the swelling was practically complete.

Fisher (private communication) found in the exudate a substance precipitable by acetic acid, insoluble in excess, which was presumed to be of a mucoid nature and which for convenience will be referred to in this paper as MP (mucoprotein) although its chemical nature is now uncertain. When the exudate is fresh MP is completely precipitated by adding half the volume of 2% acetic acid, in the form of a stringy compact coagulum. After the exudate has stood for even 2 days at 4° C., the character of this precipitate changes, becoming less compact. After a week, MP is only partly precipitable by acetic acid. These changes run parallel with changes in the ultracentrifugal sedimentation picture of the exudate (q.v.). At the same time the exudate becomes much less viscous and 'stringy', i.e. a thread is no longer formed on dipping a rod into the exudate and withdrawing it.

Analyses of nitrogen as MP, as material precipitable by 7% trichloracetic acid (SP) and as material not so precipitable (NP) (by micro-Kjeldahl), and of chloride (by tungstic acid precipitation and Volhard titration), were made on exudate from the pubic lobes of the sexual skin [Zuckerman, Van Wagenen, and Gardiner, 1938] and on serum from a number of monkeys

showing different stages of swelling. The results of these are given in Table II.

Table II. *Analysis of exudates and sera*

Monkey		MP	SP	NP	Cl mg./ml.
		N/mg./ml.			
407	Exudate	0·10	3·33	0·69	3·97
	Serum	—	9·63	0·29	3·71
408	Exudate	0·91	3·90	0·48	4·23
	Serum	—	9·83	0·26	3·83
410	Exudate	0·26	2·02	0·25	4·25
	Serum	—	7·11	0·28	3·95
404	Exudate	0·47	2·04	0·68	4·23
	Serum	—	9·03	0·40	3·74
403	Exudate	0·80	3·45	0·60	4·35
	Serum	—	9·90	0·30	3·65
406	Exudate	0·23	3·98	0·32	4·04
	Serum	—	9·19	0·28	4·01
424	Serum	—	9·43	0·28	3·95
411	Serum	—	9·86	0·31	3·38
409	Serum	—	9·71	0·28	4·01

Ultracentrifugal Sedimentation of Exudate

Fresh exudates from a number of animals showing different stages of swelling were examined in the ultracentrifuge, in a 3 mm. cell at 1060 r.p.s. by the 'diagonal schlieren' method [Philpot, 1938]. The diagrams (Plate I, Fig. 1) show three main boundaries. Two, with sedimentation constants of *ca.* $3·7 \times 10^{-13}$ and $5·2 \times 10^{-13}$ respectively, resembled normal serum proteins. The third was abnormally sharp, with a sedimentation constant of $2·7-3·7 \times 10^{-13}$ in different samples, and presumably represented the MP component. Exudate obtained by squeezing chopped cocks' combs and human umbilical cord showed similar sedimentation diagrams.

Fourfold dilution of exudate with 0·2 M NaCl caused a disappearance of the sharp boundary; exudate which had stood at 4° C. for a fortnight showed no sharp boundary; nor did an extract obtained by grinding tissue in a very early stage of swelling with Ringer solution. MP precipitated by acetic acid and suspended in saline or redissolved in alkali showed no homogeneous sedimentation boundary.

A number of other materials known to contain mucins were ultracentrifuged in order to find whether there is any constant correlation between mucin content, the abnormally sharp sedimentation boundary, and precipitability with acetic acid. These results are summarized in Table III.

Table III

Material	Character of sedimentation boundary	Precipitability with acetic acid	Stringiness
Monkey sexual-skin exudate	Abnormally sharp	+	+
Exudate from chopped cock's comb	Abnormally sharp	+	+
Exudate from chopped umbilical cord (human)	Abnormally sharp	+	+
Pig's duodenal mucin	Abnormally diffuse	−	+
Rabbit's duodenal mucin	Normal	+	+
Dog's duodenal mucin	Complex: possibly abnormally sharp boundary	(+)−	+
Saliva (human)	No sedimenting substance	+	+
Hedgehog: seminal vesicle	Complex: possibly one abnormally sharp boundary	−	−
Hedgehog: internal prostate	No sedimenting substance	−	−
Hedgehog: external prostate	Complex: possibly one or more abnormally sharp boundaries	−	+

Analysis of MP obtained by Acetic Acid Precipitations

Glacial acetic acid was added to a sample of about 30 ml. of exudate till complete precipitation was attained; the precipitate was thoroughly washed with saline and distilled water in a centrifuge tube and dried to constant weight at 108° C. in an air oven. About 80 mg. was obtained, its N content being 10·4 %. Exhaustive extraction with cold ether removed fat from the solid and raised its N content to 12·3 %. The colour produced by a specimen with Folin's phenol reagent was measured in terms of standard tyrosine; carbohydrate was estimated by the orcein method of Heidelberger and Kendall [1936], total reducing power by the Hagedorn-Jensen technique of Meyer and Palmer [1936] using zinc hydroxide precipitation. Glucosamine was very kindly estimated in a specimen by Dr. A. Neuberger, who reported: 'The substance is not completely soluble in strong HCl, and even after boiling for 3 hours in 5N HCl small bits of blackish material remained; but I don't think that they amounted to anything in weight. The glucosamine content is 2–3 %; this value excludes the possibility, in my opinion, that a typical muco-protein is concerned.' The analysis of MP is given in Table IV.

Table IV. *Analysis of MP*

	N	Folin as tyrosine	Carbohydrate (Sorensen)	Reducing power as glucose	Glucosamine
	%	%	%	%	%
Acetic acid precipitate	12·3	5·9	3·7	7·5	2–3
Ultrafilter residue	13·7	7·0	1·75	6·9	—

Ultrafiltration Experiments

The very viscous and 'stringy' nature of exudate, presumably caused by the low concentrations of MP present, together with the sharpness of the MP sedimentation boundary indicating a very low diffusion constant, suggested that the molecules of MP might be thread-like rather than spherical. Since it was clear that precipitation essentially alters its character, an attempt was made to free it from the other proteins in exudate by ultrafiltration. Serum proteins pass through a collodion Zsigmondy 'Membranfilter mittel'. Filtration of exudate through this membrane under one atmosphere of pressure gives a filtrate containing protein precipitable by trichloracetic acid, with little or none precipitable by acetic acid. The residue was successively diluted with 0·5 % sodium chloride and refiltered. The residue obtained by five such filtrations extending over 48 hours was only partly soluble in 0·2M NaCl, but the solution gave a sedimentation boundary of $S = 2·7 \times 10^{-13}$ intermediate in sharpness between the boundary shown by exudate and those of serum proteins (Plate I, Fig. 2a). No other obvious boundary was present, though there was a little very anhomogeneous material of higher sedimentation constant. More exhaustive fractionation extending over 10 days gave a sedimentation diagram resembling those of serum proteins (Plate I, Fig. 2b). The sharp boundary did not appear in a mixture of residue and ultrafiltrate which resembled in composition the original exudate (Plate I, Fig. 2c), nor was this mixture very viscous nor 'stringy'.

The soluble residue from an exhaustive course of ultrafilter fractionation was dialysed, dried, and analysed (Table IV). Its analysis indicates that it contains a considerable proportion of MP, though of changed physical properties.

Incubation of Tissue in Serum

In order to test whether the swelling of sexual skin could be reproduced *in vitro*, small pieces (50 to 200 mg.) were incubated for 22 hours at room temperature in the animal's own serum. Fresh pieces were rapidly washed in distilled water, mopped dry on filter-paper, and weighed before placing them in the serum. This process was repeated after the period of incubation. In earlier experiments only two pieces of tissue were used: in later experiments 5 pairs of pieces were taken from the areas of sexual-skin swelling, and 4 pairs of pieces of normal skin of the same animals from other parts which do not swell were treated similarly as controls. This enables the significance of differences of behaviour to be estimated.

The results were uniform. All sexual skin from which exudate could be obtained lost weight up to 35% of the original weight; all specimens of

normal skin gained in weight up to 40% of the original weight; all specimens of early stage sexual skin from which no exudate was obtainable showed a gain in weight of up to 40%. There was no significant difference between the gains of such sexual skin compared with normal skin ($P = 0.2 - 0.7$ for identity of means). Sexual skin which lost weight differed significantly from normal skin ($P < 0.01$). Loss of weight is easily explained by loss of extracellular fluid.

Histological Examination of Fresh and Incubated Tissue

After incubation the pieces of tissue described in the previous section were immediately fixed in Bouin's fluid, sectioned and stained with haematoxylin and eosin. Parallel samples, removed from the animal at the same time, were treated in the same way to serve as controls. Ten sections from each specimen were picked at random and ten fibroblasts in each section, chosen at random, were drawn with the help of a camera lucida (oil immersion lens). The areas of these cells were measured with a planimeter.

No significant differences in size of the fibroblasts were found between the tissues fixed immediately and those fixed after incubation.

DISCUSSION

Since MP is not obtainable from sexual skin except when swollen, and since it is not observable in plasma, it is probably a product of the connective tissue cells of the skin during certain phases of activity. This conclusion is supported by the cytological observation that under the influence of oestrogenic stimulation the mast-cells of the skin discharge into the intercellular spaces granules whose staining reactions are those of mucoprotein [Aykroyd and Zuckerman, 1938]. The physical properties of the substance present in exudate which gives it its peculiar character are consistent with its being a mucoprotein. Exudate from cock's comb [Hardesty, 1931] and from umbilical cord, known to contain mucins, have similar properties. On the other hand, the chemical evidence is inconclusive; the glucose content is not high. Hewitt [1938] has shown that total reducing power is an unreliable index of carbohydrate in proteins and the value here found is not higher than that quoted by Hewitt for non-mucoid proteins. A similar conclusion follows from Dr. Neuberger's glucosamine estimation. The partial success of ultrafiltration confirms the ultracentrifuge in indicating that the molecules are thread-like, and suggests that this might be a successful method for obtaining the substance in a pure and native state, if conditions could be found under which it is stable.

For practical purposes sexual-skin exudate can be regarded as the tissue fluid of the skin when it is swollen. The composition of exudate does not vary much with the phase of swelling. The most marked differences from

serum are: the presence of MP; the lower concentration of serum-like proteins; higher non-protein nitrogen; and, except for the exudate from a monkey (406) in a very late stage of swelling, higher total chloride. The excess of chloride is too high to be accounted for by Donnan effects [cf. Flexner, 1937, 1938; Hodgson, 1938].

No definite conclusion can be drawn as to the functional significance of the MP and the high chloride concentration. The low concentration of MP, and physical considerations based on the ultracentrifuge results, make it unlikely that it contributes appreciably to the swelling process by attracting water. The high chloride concentration can attract water by osmosis provided the excess chloride is free and not held by adsorption on a few large molecules. The energy needed to concentrate the chloride under such conditions can reasonably be supposed to come only from the cells, and if the latter have no direct access to the capillaries, they can only concentrate chloride at the expense of the tissue fluid itself. Hence a high concentration in tissue fluid can only be maintained by means of previously accumulated reserves in the cells. This conclusion is not necessary, however, if the cells have direct access to the capillaries. The hypothesis that swelling is due to the osmotic pressure of excess free chloride is therefore a complicated one, though it receives some support from the freezing-point measurements of Fisher and Zuckerman [1937].

If the capillaries have normal blood-pressure and permeability, the fairly high concentration of protein in exudate is sufficient alone to maintain the swelling. If these proteins, which with the exception of MP resemble plasma proteins, have actually come from plasma, there must be abnormal capillary permeability which must further contribute to the swelling. There is therefore no need to invoke the high chloride or the MP to explain the swelling, though such evidence as exists suggests that the former does in fact play a part.

Summary

Experiments were designed to throw light on the process of swelling of the sexual skin of immature female rhesus monkeys.

1. The 'serum protein', 'mucoprotein', non-protein nitrogen, and chloride contents of sexual skin exudate and of serum were compared at different stages of swelling.

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3. Ultracentrifuge and chemical observations were made on various other mucin-containing animal fluids.

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4. Some chemical observations were made on the 'mucoprotein' fraction of exudate.

5. Observations were made on the behaviour of swollen and unswollen sexual skin and of non-sexual skin incubated in serum; changes in weight and histological appearance were measured.

The data collected are discussed in relation to the probable mechanism of sexual skin swelling.

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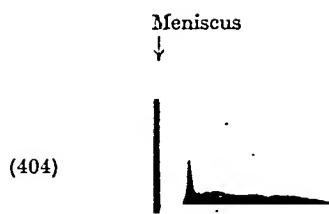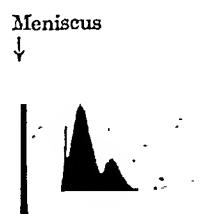
FIG. 1. Sedimentation diagrams of pubic lobe exudate showing sharp (MP) boundary and normal (SP) boundaries.

FIG. 2. Sedimentation diagrams of ultrafilter residues. See text *'Ultrafiltration Experiments'*.

SPECIES VARIATION IN THYROTROPHIC, GONA-DOTROPHIC, AND PROLACTIN ACTIVITIES OF THE ANTERIOR HYPOPHYSEAL TISSUE

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DURING the past few years it has become clear that anterior pituitary glands from different species of animal contain different proportions of physiologically active substances. Loeb and Friedman [1933], in comparing the anterior pituitary glands of various species, distinguished two groups according to their contents of gonadotrophic and thyrotrophic hormones. In one class, in which they placed ox, sheep, and pig, the gland possessed high thyrotrophic activity and high gonadotrophic activity of the type considered to be due to the 'luteinizing hormone' of the gonadotrophic complex. In the second class, in which they placed the cat, rat, guinea-pig, and rabbit, the pituitary gland contained little thyrotrophic hormone but a larger proportion of the follicle-stimulating constituent of the gonadotrophic complex. In this second class it is possible also to place the horse and adult man [Rowlands, 1936; Henderson and Rowlands, 1938; Cope, 1938]. An association between the thyrotrophic and the luteinizing hormone has also been observed by Greep [1935] and Rowlands [1936].

The relative gonadotrophic activities of extracts of the pituitary gland of a large number of species were determined by Hill [1934], who found that the pituitary gland of the horse is the most active among those of the common domestic species. This observation is in agreement with the results of Bugbee, Simmonds, and Grimes [1931], Wallen-Lawrence and van Dyke [1931], and Hellbaum [1933], who also found that horse pituitary glands are more gonadotrophically active than those of either pig or sheep. Ox pituitary gland apparently contains less gonadotrophic hormone than any of these [Hill, 1934; Noble, Rowlands, Warwick, and Williams, 1939].

In a comprehensive study of the content of the lactogenic and thyrotrophic hormones in the anterior pituitary glands of various species, Reece and Turner [1937] concluded that a marked species variation exists in the amount of the lactogenic hormone, and arranged the pituitary glands of the females of the species examined in the following descending order of potency—ox, guinea-pig, rat, rabbit, mouse, oestrous cat, and anoestrous cat. Those of the male sex were arranged in a similar order, viz. guinea-pig, rat, rabbit, and cat.

Definite cyclical changes in the activity of the anterior pituitary gland, causing variations in the quantity of the various hormones secreted by the gland, have been described. It has been found that the amount of hormone contained in the pituitary gland varies with the age of the animal, and may fluctuate during the oestrous cycle. Bates, Riddle, and Lahr [1935] showed that differences occur in the thyrotrophic, gonadotrophic, and lactogenic capacity of the pituitary glands of cows of different ages, and Reece and Turner [1937] found similar differences in the thyrotrophic and lactogenic activities of the same species. The amount of lactogenic hormone was at a maximum in non-lactating dairy cows and was lowest in the pituitary gland of the foetus. Hellbaum [1935], investigating the gonadotrophic activity of the horse pituitary gland (mares, stallions, and geldings), observed activity to be greatest in the glands of old mares and young geldings, the greater activity at this time apparently being due to a higher proportion of the follicle-stimulating hormone.

A knowledge of the hormonal constitution of the anterior lobe of the pituitary gland of different species is of general value, for, in the preparation of extracts, it is obviously desirable to use, as starting material, tissue which contains a high proportion of the particular hormone required. In large-scale work the differences in hormone content associated with age, sex, and general condition have usually to be overlooked, although by selection of the type of pituitary gland it is possible to ensure that a starting material with high activity is used.

Pituitary glands suitable and available for large-scale work are generally limited to those from ox, sheep, pig, horse, and man, and the present investigation was undertaken to determine the relative proportions of thyrotrophic hormone, gonadotrophic hormones, and prolactin present in these tissues and extracts of them. Assays were made directly on the acetone-desiccated tissue and on crude alkaline extracts. The crude extract was then separated into material soluble at a pH around 5, and material spontaneously precipitating at the same pH, a crude separation of the prolactin from the thyrotrophic and gonadotrophic hormones thus being effected. In this way it was possible to determine, not only the relative activities of the different pituitary tissues themselves, but also the relative amounts of solid material which might be expected to accompany the physiologically active substances in the course of a simple fractionation.

<div align="center">METHODS</div>

<div align="center">*Physiological*</div>

In this investigation it has not been possible to construct a dose-response curve for each type of physiological activity exhibited by all the different fractions. In those instances in which such a curve was not con-

structed the physiological results were expressed in terms of defined units by reference to a standard dose-response curve. It is clear that unless the dose-response curve is the same for all the pituitary extracts used (and such is not the case) and does not vary with the season of the year, an error will thus be introduced. An attempt has been made to minimize such an error by choosing test doses of the substance under assay which contain very nearly one unit of physiological activity, and by carrying out a number of different assays at different times of the year, and averaging the results.

In nearly all instances assay results were based on the average response of a group of 10 animals, although in a few experiments the groups contained only 5 animals.

As International Standard preparations of the different hormones, standardized in terms of International Units,[1] are not yet available we were compelled, in most instances, to express our results in terms of arbitrarily defined units of biological activity. The International Unit of prolactin (see section IV, para. 6, of the above bulletin) is the same as the Riddle Unit which has been employed in this paper for the assay of prolactin. The International Unit of thyrotrophin (see section III, para. 3) is also about equal to the unit of the thyrotrophic activity described by Rowlands and Parkes [1934].

Assay of thyrotrophic hormone. This was carried out by the method of Rowlands and Parkes [1934]. The results are expressed in terms of the units defined by these authors, i.e. a unit is that amount of active substance which, when injected subcutaneously into an immature guinea-pig once daily for 5 days, causes the thyroid glands to double their weight. For the purpose of expressing physiological results in terms of units, the assay curve shown in Fig. 1 was used.

Assay of prolactin. This was determined on the basis of the pigeon crop gland response, according to the systemic method described by Rowlands [1937]. The results are expressed in Riddle Units, this being made possible by comparing the results with an assay curve (Fig. 2) constructed by our methods with a preparation of prolactin prepared and standardized by Drs. Riddle and Bates.

Assay of gonadotrophic hormone. Two methods were used. The first was based on the ability of the extracts to increase the weight of the ovary of the immature rat.

A unit of activity was arbitrarily chosen as that amount of extract which, when administered subcutaneously to a group of female rats weighing 40–50 g., once daily for 5 days, increased the total weight of the ovaries from an average of 10 mg. to an average of 40 mg. The results

[1] *Bulletin of the Health Organisation of the League of Nations,* 1938, vol. vii, no. 32.

were expressed in terms of units with the aid of the assay curve shown in Fig. 3. As the dose-response curve obtained with extracts from some species becomes asymptotic to a line representing a very small increase

UNITS
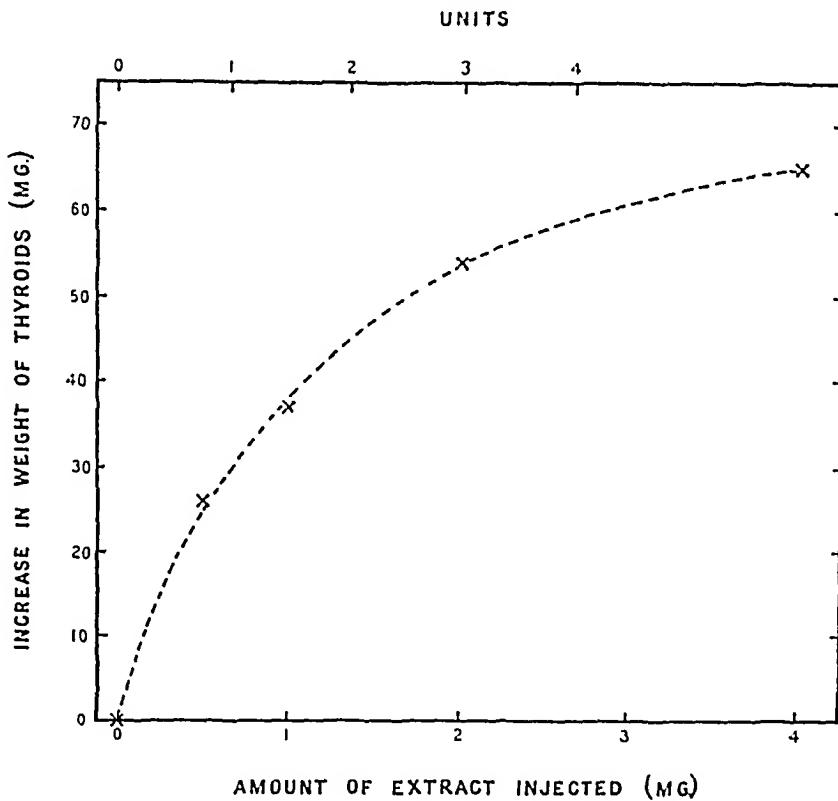
Fig. 1. Assay curve for thyrotrophin (AP52D₁).

in ovary weight, care was taken to ensure that the test dose was small enough to give a result falling on the steep part of the curve.

The second method of assay of gonadotrophic activity was based on the ability of the extracts to cause ovulation in the oestrous rabbit. The unit of activity was defined as that amount of extract which, when injected intravenously in one dose, would cause ovulation in 50% of a group of 10 oestrous rabbits within 24 hours. The assay curve shown in Fig. 4 was used for the assessment of results.

It is probable that these two different methods of assay for gonadotrophic activity do not determine the same active substance. How far the results are determined by the relative proportions of 'follicle-stimulating' hormone and 'luteinizing' hormone cannot be discussed here, but it should be mentioned that there is no reason to suppose that either of our methods of assay determines only one or the other of these two presumed pituitary substances.
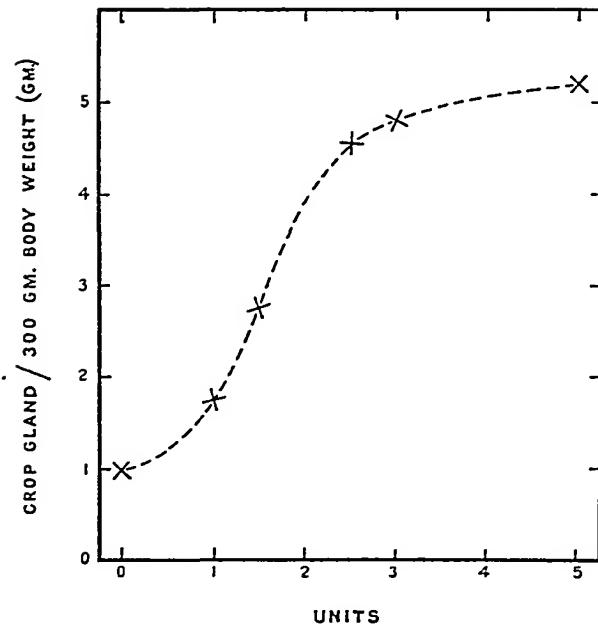
FIG. 2. Assay curve for prolactin.
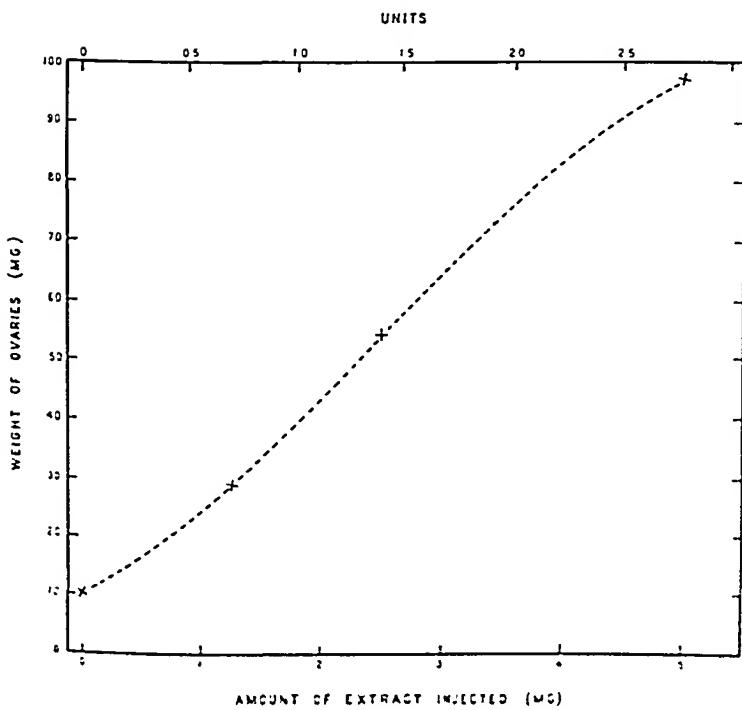
FIG. 3. Assay curve for gonadotrophin (AP70B) (Rat Units).
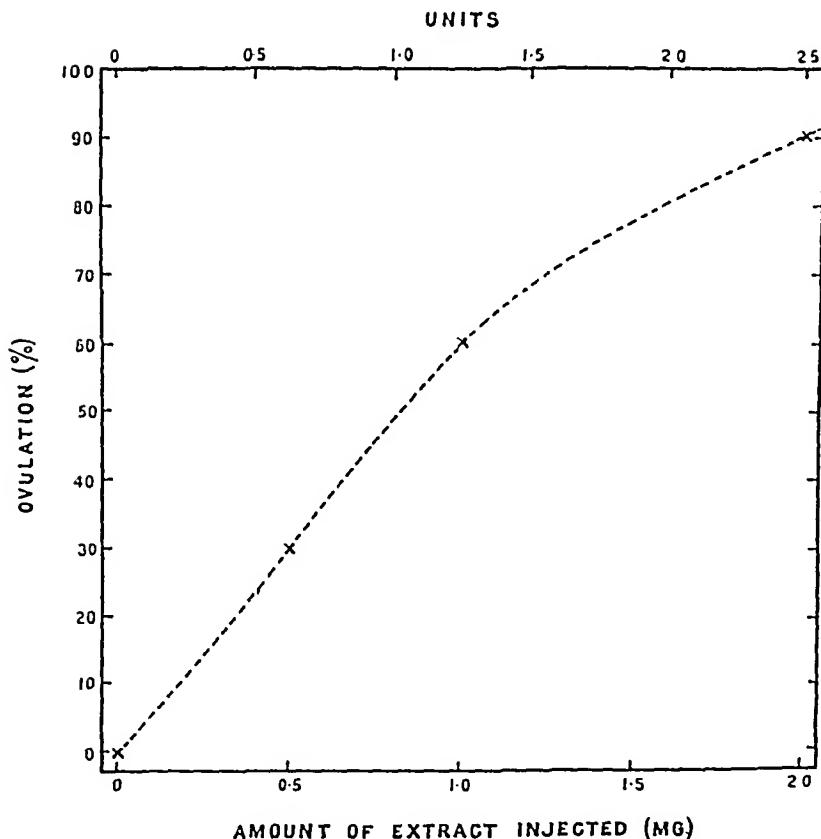
Fig. 4. Assay curve for gonadotrophin (AP15B) (Rabbit Units).

Chemical

All the extracts used in the present investigation were prepared from acetone-desiccated pituitary tissue. In most instances the pituitary glands were received at the laboratory in a frozen condition. The anterior lobes were dissected from posterior lobe tissue and minced into a large volume of acetone (at least 20 ml./g. of fresh tissue) at room temperature. The glands were subsequently washed with fresh acetone and dried *in vacuo*. In a few experiments undissected whole pituitary gland tissue was used, but in most instances the posterior lobes were removed before desiccation.

Many of the ox pituitary extracts were prepared from commercial acetone-desiccated anterior lobe tissue. No knowledge was available as to the period elapsing between the death of the animals from which the glands were obtained and removal of the pituitary gland, nor was there any clear indication as to the time during which the glands were allowed to remain at room temperature before being frozen. In a few instances ox anterior pituitary glands were available which had been frozen in solid carbon-dioxide snow immediately after removal from the carcass 1 hour

or less after the death of the animal. These fresh glands were dissected while still frozen and minced into ice-cold acetone. The tissue was subsequently extracted thoroughly with cold acetone, and dried *in vacuo*.

The processes of extraction and fractionation were carried out according to the scheme depicted in Fig. 5. These processes, with the exception of dialysis, were carried out at room temperature.

Acetone Desiccated Pituitary Tissue

```
                    |
     ┌──────────────┴──────────────────┐
  'B' Fraction                      'A' Fraction
  Soluble in alkaline               Insoluble in alkaline
  extraction fluid.                 extraction fluid.
     |                                 |
  ┌──┴───────────────────┐     ┌───────┴──────────────────┐
'C' Fraction                  'D' Fraction
Spontaneously precipitates    Soluble in aqueous
from aqueous solution over    solution between pH 3 and pH 6.
a pH range lying between
pH 3 and pH 6.
     |                                 |
  ┌──┴──────────┐              ┌───────┴──────────┐
'F' Fraction   'G' Fraction   'D₁' Fraction   'D₂' Fraction
Insoluble in   Soluble in     Soluble after   Insoluble after
70% alcohol in 70% alcohol in prolonged       prolonged
the presence   the presence   dialysis.       dialysis.
of 0·1% NaCl   of 0·1% NaCl
at pH 6.       at pH 6.
```

Fɪɢ. 5. The relationship of the different fractions used in the present investigation. The 'C' fraction contains most or all of the prolactin recovered from the 'B' fraction, while the 'D' fraction contains gonadotrophic and thyrotrophic activities. The 'G' fraction is equivalent to the prolactin of Bates and Riddle [1935].

All pH determinations were made colorimetrically and are therefore only approximately correct. Specimens of solutions containing alcohol were diluted with water before addition of the indicator.

Precipitates were dried by the following process: The pH of the solution was adjusted to about pH 5, and poured into 4–5 volumes of alcohol (Industrial Methylated Spiritis-Methcol) with stirring. The precipitate was allowed to settle, usually overnight, and the supernatant fluid decanted. The precipitate was then stirred with fresh alcohol, and again allowed to settle. Three or four washings with alcohol were thus effected. The final precipitate was filtered on a Buchner funnel and washed on the filter with three or four portions of dry ether. Immediately the excess ether had drained off, the funnel was placed in a desiccator which was at once evacuated. In this way it was possible to obtain the different pituitary fractions in the form of light dry powders.

Preparation of 'B' fraction. Four types of alkaline extractant were used for the preparation of the 'B' fractions: (*a*) N/20 NaOH, (*b*) dilute

NaOH at pH 8–10, (c) aqueous pyridine solution, or (d) alkaline aqueous alcohol solution.

(a) In a few experiments alkaline extraction was carried out by stirring the desiccated tissue with N/20 NaOH. Two or three successive extractions were performed and the combined extracts adjusted to about pH 5 by the addition of dilute HCl; 4 volumes of alcohol were then added and the precipitate dried.

(b) More frequently, aqueous alkaline extraction was effected by stirring the desiccated tissue with 10–15 times its weight of water, the pH of which was adjusted to 8·5–10 by the addition of a small amount of NaOH solution, and maintained at the initial pH by the frequent addition of further small amounts of alkali. In general three or four successive extractions were performed, the pH of successive extracting fluids rising from pH 8·5 to pH 10. The combined extracts were adjusted to about pH 5, and precipitated and dried in the usual way.

(c) The acetone desiccated tissue was stirred overnight with 20 times its weight of 50% aqueous pyridine, one extraction only being made. The soluble material was precipitated by the addition of 4 volumes of alcohol, and dried in the usual way. This method is essentially that of Fevold, Hisaw, and Leonard [1931].

(d) The method described by Bates and Riddle [1935] was also used. The desiccated tissue was stirred with 10–20 times its weight of 60% or 70% aqueous alcohol solution, the pH of which was adjusted to 8·5–10 by the addition of dilute NaOH. The pH was maintained at its initial value by the frequent addition of small amounts of NaOH solution. Three or four successive extracts were made, the pH rising from pH 8·5 for the first extract to pH 10 for the last. The combined extracts were adjusted to approximately pH 5 by the addition of HCl, and 3 volumes of alcohol were added. The precipitate was dried in the usual way. In a few experiments on horse pituitary gland, different strengths of aqueous alcohol varying between 20% and 70% were used for extraction, but in most experiments with aqueous alcohol, 60% or 70% alcohol was used.

The residue which remained after the extraction of the 'B' fraction according to the above methods was stirred with alcohol and dried in the usual way. This constituted the 'A' fraction.

Preparation of 'C' fraction. Two main methods were used for the separation of the 'C' fraction from the 'B' crude extract: (a) precipitation of the 'C' fraction at the point of maximum precipitation (about pH 5·5) from water [cf. Riddle, Bates, and Dykshorn, 1933]; (b) precipitation from 0·5% Na_2SO_4 solution at about pH 3·5 [cf. Bates and Riddle, 1935], followed by precipitation from water at about pH 5·5.

With both methods the initial step was to dissolve the 'B' fraction by

stirring in water at about pH 8·5, the pH being maintained by the frequent addition of small amounts of NaOH. Any insoluble material was centrifuged off, re-extracted a second or third time, and finally discarded. The final concentration of the solution of 'B' fraction was usually about 0·5 g. per 100 ml.

(*a*) The pH of the solution was adjusted to the point of maximum precipitation (about pH 5·5) and the precipitate centrifuged off, redissolved at pH 8, and again precipitated at pH 5·5. This process of precipitation at pH 5·5, followed by re-solution at pH 8 and reprecipitation, was carried out 4–5 times altogether. Occasionally it was necessary to add a trace of NaCl to ensure proper precipitation at pH 5·5 in the later stages. It was sometimes observed that the point of maximum precipitation moved slightly towards the alkaline side as the reprecipitations proceeded. The final pH 5·5-insoluble material was dried with alcohol and ether, and constituted the 'C' fraction.

(*b*) 0·5% Na_2SO_4 was added to the solution of the 'B' fraction and the pH adjusted to the point of maximum precipitation (about pH 3·5 in this case) by the addition of dilute H_2SO_4. The precipitate was redissolved at pH 8 and reprecipitated a number of times at pH 3·5 in the presence of Na_2SO_4. If any of the supernatants gave a precipitate on adjusting too close to pH 5·5, this precipitate was added to the main precipitate at pH 3·5. The final pH 3·5-insoluble material was then reprecipitated a large number of times at pH 5·5 according to the method described in section (*a*). The final precipitate was dried with alcohol and ether, and constituted the 'C' fraction.

In one instance prolactin was prepared from sheep pituitary glands by the method of Lyons [1937].

Preparation of 'D' fraction. (*a*) The pH 5·5-soluble material from section (*a*) in the preparation of 'C' fraction constituted one type of 'D' fraction. This was dried in the usual way.

(*b*) The other type consisted of the pH 3·5-soluble material from section 'C' (*b*). This was precipitated by the addition of 4 volumes of alcohol, and dried in the usual way. The 'D' fraction obtained by this method contained some Na_2SO_4. This was removed by dialysis against distilled water at 2° C. The material which remained in solution after prolonged dialysis constituted the 'D₁' fraction. These were dried in the usual manner.

(*c*) In some instances a 'D' fraction was prepared directly from the acetone desiccated tissue by exhaustive extraction with acetate buffer at pH 4·6 [cf. Wallen-Lawrence and van Dyke, 1931]. The extracts were precipitated with alcohol and dried, and are referred to as 'van Dyke' preparations.

Preparation of 'F' and 'G' fractions. In a few instances the 'C' fraction was divided into fractions which are either soluble or insoluble

in 70% alcohol at pH 6 in the presence of 0·1% NaCl according to the method described by Bates and Riddle [1935]. The soluble fraction (the prolactin of Bates and Riddle) was called the 'G' fraction, and the insoluble material the 'F' fraction.

RESULTS

Relative biological activities of acetone desiccated anterior pituitary tissues.

As desiccation in acetone was the first treatment to which the fresh pituitary tissue was subjected, and as the resulting powder constituted the starting material for all subsequent extractions, biological assays for gonadotrophic, thyrotrophic, and lactogenic activity were performed on samples of dried tissue from the five different species. The results are summarized in Table I. A comparison is also shown in Table I of the activities of desic-

Table I. *The hormonal activity of the anterior pituitary gland of various species.*

		Species examined					
Hormonal activity		Man	Horse	Pig	Sheep	Ox. Commercial dried gland	Ox. Freshly dried gland
Thyrotrophin	Rowlands-Parkes units/ 100 g. acetone dried gland	—	1,070	9,100	5,500	3,700	6,750
	Relative activity (pig = 100)	[26]*	12	100	60	41	74
Prolactin	Riddle units/ 100 g. acetone dried gland	59,000	6,450	7,520	160,000	32,000	150,000
	Relative activity (sheep = 100)	37	4	5	100	20	94
Gonadotrophin. Immature rat test	Rat units/100 g. acetone dried gland	355,000	107,000	5,500	8,000	very small	very small
	Relative activity (man = 100)	100	30	1·5	2·5	[0·19]†	[0·18]‡
Gonadotrophin. Rabbit ovulation test	Ovulation units/ 100 g. acetone dried gland	213,000	91,000	143,000	140,000	29,000	91,000
	Relative activity (man = 100)	100	43	67	66	13	43

* Calculated from value for van Dyke 'D' fraction
† Calculated from value for 50% pyridine 'B' fraction.
‡ Calculated from value for van Dyke 'D' fraction.

cated fresh ox anterior lobe and commercial desiccated ox anterior pituitary tissue; the latter results illustrate the importance of having as starting material dried powder prepared from fresh pituitary glands. The results in Table I and Fig. 6 show that the pituitary gland of the pig contains the most thyrotrophic activity per unit weight, and is followed in descending order of potency by that of ox, sheep, horse, and man. The
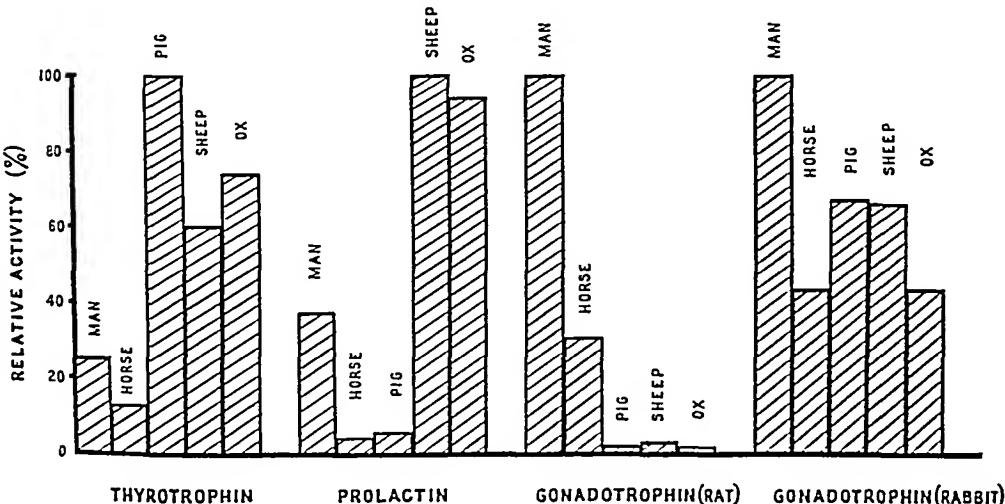
Fɪɢ. 6. Relative hormonal activities of acetone-dried anterior pituitary tissues from different species.

differences in the prolactin content of the pituitary gland of the same five species is even greater than the differences observed in the content of the thyrotrophic hormone. Pituitary tissues from sheep and ox were found to be the most active; the human pituitary tissue contained about 40% of this amount of prolactin, and that of the horse and pig only 4–5% of this amount.

The gonadotrophic activity of the human pituitary gland was found to be about 3 times as great as that of horse pituitary gland in stimulating ovarian growth in the immature rat, but only twice as great in causing ovulation in the oestrous rabbit. When expressed in rat units it is seen that the relative activity of the pituitary gland of the sheep is only 2·5% that of the human pituitary gland, that of pig only 1·5%, and that of ox even smaller. This remarkable species difference is to be contrasted with the far greater uniformity in the content of the ovulation-producing substance, expressed in rabbit units, in the same specimens of desiccated pituitary tissue. The value of the expression

$$\frac{\text{rat gonadotrophic units} \times 100}{\text{rabbit gonadotrophic units}}$$

is 170 for human pituitary tissue, 120 for the horse, only 5 for the pig,

4 for the sheep, and probably about 1 or less for ox anterior pituitary tissue. These enormous variations in the ratio of the activities determined by the two types of gonadotrophic assay suggest that the activities of two different types of gonadotrophic substances are being determined.

Thyrotrophin.

The data relating to the activities of the different fractions examined are shown in Table II. The results show that with ox, pig, and horse tissues 50% aqueous pyridine, alkaline aqueous alcohol, and dilute aqueous alkali are all good extractants, although 50% aqueous pyridine, and alkaline aqueous alcohol were not such effective extractants for sheep pituitary tissue.

With pig and horse tissues, 'D' fractions prepared by aqueous extraction followed by 'isoelectric' precipitation of the 'C' fraction are similar in activity to corresponding fractions prepared by the van Dyke method, about 50% of the thyrotrophic activity in the acetone desiccated gland appearing in these fractions. On the other hand, in the case of ox and sheep glands the van Dyke method yields a far more potent 'D' fraction than does isoelectric precipitation of the 'C' fraction from an aqueous alkaline extracting medium, although extraction of the commercial ox tissue by alkaline aqueous alcohol followed by isoelectric precipitation yielded a 'D' fraction approximately equal in activity to that obtained by the van Dyke method.

On the whole the van Dyke acid buffer and the Bates-Riddle aqueous alkaline alcohol procedures seem to be the most satisfactory for the preparation of thyrotrophic extracts.

Prolactin.

Table III indicates the yields and activities of the different fractions examined. The results show that the Bates-Riddle alkaline aqueous alcohol method is satisfactory for the preparation of prolactin from ox pituitary tissue, although in our hands it was less effective with sheep and horse pituitary tissue. Aqueous extraction followed by simple isoelectric precipitation of the prolactin fraction was satisfactory with fresh ox, pig, and human pituitary tissues, but less effective with commercial ox and sheep glands. The Lyons [1937] method gave a highly active prolactin preparation from sheep tissue, though the total recovery of activity was not good.

It should be noted that, with horse tissue, aqueous alkaline extraction was much more effective than extraction with alkaline aqueous alcohol. This observation is of interest in view of the results given in the next section.

Table II. *Thyrotrophic activity*

Species	Fraction	Method of preparation	No. of extracts	Yield of dried material %	Units/100 g. of dried gland	Relative activity (dried gland = 100)
Ox (fresh)	—	Acetone desiccation	2	100·0	6,750	100
	D	Aqueous pH 8–10 ppt. pH 5·5	2	2·4	1,710	25
	D	van Dyke	2	12·5	6,800	100
	A+C	van Dyke (prolactin fraction)	2	77·0	930	14
Ox (commercial)	—	Acetone desiccation	2	100·0	3,700	100
	B	50% aq. pyridine	5	10·3	4,690	127
	,,	N/20 NaOH	5	10·6	2,900	80
	,,	70% alcohol	5	16·7	3,130	85
	D	Aqueous pH 8–10 ppt. pH 5·5	2	2·7	730	20
	,,	Aqueous alcohol ppt. pH 3·5	5	9·5	1,780	48
	,,	van Dyke	2	3·6	1,870	51
	D_1	Aq. pH 8–10, &c., dialysed	5	1·13	1,240	34
	D_2	Aq. pH 8–10, &c., ppt. after dialysis	5	0·43	85	2
Pig	—	Acetone desiccation	1	100·0	9,100	100
	B	50% aq. pyridine	1	16·0	5,920	65
	D	Aqueous pH 8–10 ppt. pH 5·5	5	8·8	5,580	61
	,,	van Dyke	1	10·0	5,260	58
Sheep	—	Acetone desiccation	1	100·0	5,500	100
	B	50% aq. pyridine	1	9·5	665	12
	,,	70% alcohol	1	5·0	450	9
	D	Aqueous pH 8–10 ppt. pH 5·5	3	5·7	1,230	22
	,,	van Dyke	4	7·0	3,900	71
	—	Acetone desiccation	1	100·0	1,070	100
Horse	B	50% aq. pyridine	1	13·0	615	53
	,,	Aqueous at pH 8–10	1	22·9	1,530	140
	D	Aqueous pH 8–10 ppt. pH 5·5	3	5·4	420	40
	,,	van Dyke	1	6·0	570	53
Man	,,	,,	1	9·25	1,380	—

Table III. *Prolactin activity*

Species	Fraction	Method of preparation	No. of extracts	Yield of dried gland %	Units/100 g. of dried gland	Relative activity (dried gland =100)
Ox (fresh)	—	Acetone desiccation	2	100	150,000	100
	C	Aqueous pH 8–10 ppt. pH 5·5	2	10·9	63,700	42
	„	Aqueous alcohol ppt. pH 5·5	2	4·75	38,700	26
	A+C	van Dyke	2	77·0	106,500	71
Ox (commercial)	—	Acetone desiccation	2	100	32,000	100
	B	Aqueous alcohol	5	16·7	14,500	45
	C	Aqueous pH 8–10 ppt. pH 5·5	2	7·6	3,160	10
	„	Aqueous alcohol ppt. pH 3·5	5	11·6	18,900	59
	A+C	van Dyke	3	80·2	20,200	63
	F	Bates-Riddle	2	1·9	1,610	5
	G	„	3	1·17	7,680	24
Pig	—	Acetone desiccation	1	100	7,520	100
	C	Aqueous pH 8–10 ppt. pH 5·5	5	14·5	4,450	59
Sheep	—	Acetone desiccation	2	100	160,000	100
	B	Aqueous alcohol	1	6·6	27,500	17
	C	Aqueous pH 8–10 ppt. pH 5·5	2	8·9	15,600	10
		Lyons [1937]	1	0·4	5,190	3
	A+C	van Dyke	4	84·9	175,000	110
	„	„	1	80·0	186,000	100
	C	van Dyke+ Aq. pH 8–10 ppt. pH 5·5	1	15·6	89,000	48
	A	van Dyke residue from above	1	65·6	50,000	27
Horse	—	Acetone desiccation	1	100	6,450	100
	B	Aqueous pH 8–10	3	25·1	4,600	71
	„	Aqueous alcohol	2	8·8	442	7
Man	—	Acetone desiccation	1	100	59,000	100
	C	Aqueous pH 8–10 ppt. pH 5·5	1	14·5	22,000	37
	A+C	van Dyke	1	73·2	81,000	137

Gonadotrophin.

Assay on the immature rat. The results for gonadotrophic activities, using the growth of the ovary of the immature rat for assay, are shown in Table IV. The results show that only about one-quarter of the initial activity is found in the 'D' fraction prepared by aqueous extraction of the desiccated gland followed by isoelectric precipitation of the prolactin fraction. On the other hand, with the van Dyke method of extraction,

Table IV. *Gonadotrophic activity assayed in rat units*

Species	Fraction	Method of extraction	No. of extracts	Yield of dried gland %	Units/100 g. of dried gland	Relative activity (dried gland = 100)
Ox (commercial)	B	50% aqueous pyridine	2	10	675	—
	D₁	Aqueous pH 8–10 ppt. of 'C' at pH 5·5	1	1·43	70	—
	,,	Aqueous alcohol ppt. at pH 3·5	1	1·3	90	—
Sheep	—	Acetone desiccation	1	100	8,000	100
	B	70% alcohol	3	7·2	1,370	17
	D	Aqueous pH 8–10 ppt. of 'C' at pH 5·5	3	6·1	1,760	22
	,,	van Dyke	4	7·0	7,470	93
Pig	—	Acetone desiccation	1	100	5,500	100
	D	Aqueous pH 8–10 ppt. of 'C' at pH 5·5	4	8·9	1,620	29
	,,	van Dyke	1	10·0	10,000	125
Horse	—	Acetone desiccation	1	100	107,000	100
	B	N/20 NaOH	1	47·0	47,000	44
	,,	50% pyridine	2	12·5	23,000	22
	,,	Aqueous at pH 8–10	5	26·2	63,100	59
	,,	60% alcohol	2	8·3	21,500	20
	,,	70% alcohol	1	8·8	11,400	11
	D	Aqueous pH 8–10 ppt. of 'C' at pH 5·5	6	5·3	26,800	25
	,,	van Dyke	1	6·0	30,000	28
Man	—	Acetone desiccation	2	100	355,000	100
	D	van Dyke	1	9·25	138,000	39
	A+C	van Dyke (residue)	1	73·2	1,500	0·3

approximately quantitative recovery of activity in the 'D' fraction was obtained in the case of glands from the sheep and pig, the activities of which are relatively low in this respect, whereas only about one-third of the total activity was recovered from horse and human tissues, which are much more active in this respect.
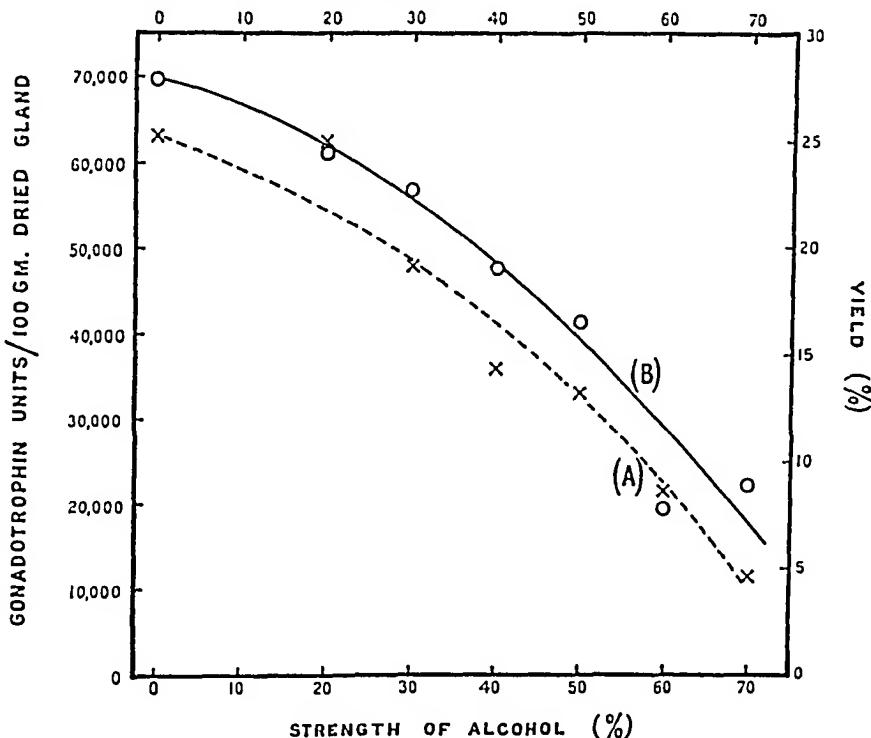
Fig. 7. Total gonadotrophic activity (rat ovary assay)—curve A, and solid material—curve B, extracted from acetone desiccated horse pituitary tissue by different strengths of alkaline aqueous alcohol solution.

It was of particular interest to find that in the case of horse tissue, alkaline aqueous alcohol (70%) is a very poor extractant of this type of gonadotrophic activity, compared with simple aqueous alkali. A further investigation of this point was made by extracting a number of different samples of desiccated horse tissue from the same batch of material with alkaline aqueous alcohol containing different percentages of alcohol. The results, which are given graphically in Fig. 7, show that the amount of solid material extracted and the total amount of activity recovered rapidly decrease with increasing concentrations of alcohol, the total activity recovered being approximately proportional to the amount of solid material extracted. It is clear from these results that aqueous alkali is a better extractant for horse tissue than is aqueous alkaline alcohol of any strength.

Assay on the oestrous rabbit. The results of assays in which oestrous

rabbits were used are given in Table V. It will be seen that there is little to choose between the methods used, though it should be pointed out that simple aqueous alkali is apparently an excellent extractant for this type of gonadotrophic activity. Seventy per cent. alkaline aqueous alcohol also proved to be an effective extractant for ox and horse pituitary tissues.

Table V. *Gonadotrophic activity in rabbit units*

Species	Fraction	Method of extraction	No. of extracts	Yield of dried gland %	Units/100 g. of dried gland	Relative activity (dried gland = 100)
Ox (fresh)	—	Acetone desiccation	1	100	91,000	100
	D	van Dyke	1	11·9	39,700	44
Ox (commercial)	—	Acetone desiccation	1	100	28,600	100
	B	50% pyridine	2	10·2	22,400	78
	,,	N/20 NaOH	1	1·7	11,200	39
	,,	70% alcohol	1	5·3	10,600	37
	,,	60% alcohol	2	21·2	26,500	93
	D	Aqueous alcohol ppt. at pH 3·5	1	7·1	3,550	12
	D_1	,,	1	1·05	2,625	9
	D_2	,,	1	0·28	190	1
Pig	—	Acetone desiccation	1	100	143,000	100
	D	Aqueous pH 8–10 ppt. at pH 5·5	1	5·75	95,800	67
	,,	van Dyke	1	10·0	71,400	50
Sheep	—	Acetone desiccation	1	100	140,000	100
	B	Aqueous alcohol	3	7·2	38,000	27
	D	van Dyke	2	6·5	59,500	43
Horse	—	Acetone desiccation	1	100	91,000	100
	B	Aqueous pH 8–10	1	28	80,000	90
	,,	70% alcohol	1	8·85	50,000	56
	D	Aqueous pH 8–10 ppt. at pH 5·5	3	3·6	45,300	50
	,,	van Dyke	1	6·0	60,000	66
Man	—	Acetone desiccation	1	100	213,000	—

Completeness of separation of different hormones.

Table VI gives the results of some experiments carried out to determine how far thyrotrophic and gonadotrophic activities were present in the 'C' (prolactin) fraction, and conversely what was the prolactin activity of the

'D' fractions. The assays show that extraction with 60% or 70% alcohol followed by separation of the 'C' and 'D' fractions at pH 3·5 in the presence of 0·5% Na_2SO_4 resulted, in the case of ox pituitary extracts at least in a complete separation of the prolactin activity from the thyrotrophic

Table VI. *The completeness of separation of different hormones*

Activity tested	Species	Fraction	Method of preparation	Daily dose giving no observable effect mg.	Activity in 'C' or 'D' fraction compared with that in corresponding 'D' or 'C' %
Thyrotrophin	Ox	32 C	Bates-Riddle	20	0
		37 C		20	0
		50 C		20	0
		52·1 C		20	0
		52·2 C		20	0
		48 C	Aqueous extraction	—	41
	Pig	43 C		—	14
Prolactin	Ox	32 D_1	Bates-Riddle	20	0
		37 D_1		20	0
		50 D_1		20	0
Gonadotrophin (rat ovary)	Horse	39 C	aqueous extraction pH 8–10 ppt. at pH 5·5	—	20·5
		41 C		—	15·9
		54·1 C		—	1·8
		58·1 C		—	0·6
Gonadotrophin (oestrous rabbit)	Ox	32 G	Bates-Riddle	40	0
		37 C		100	0
	Horse	39 C	Aqueous extraction	—	8·5
		41 C		—	7·0

and gonadotrophic activities. 'Complete' is, of course, a relative term. Aqueous extraction followed by separation of the 'C' and 'D' fractions at pH 5·5 did not give complete separations of the different types of activity.

Distribution of solid matter in the different fractions.

Table VII gives the amounts of solid matter (expressed as % of dried gland) found, on the average, in the 'C' and 'D' fractions of the different species, prepared by aqueous extraction. The final column gives the

Table VII. *Distribution of solid matter between the 'C' and 'D' fractions of the different species*

	Solid matter as % of dried gland, present in:		
Species	'C' fraction	'D' fraction	'C' and 'D' fractions combined
Ox	9·3	2·6	11·9
Sheep	8·3	6·1	14·4
Pig	14·5	8·8	23·3
Horse	14·3	5·3	19·6

total of the solid in the two fractions combined, and indicates that pig pituitary gland has about twice as much aqueous extractable matter as ox pituitary gland. Sheep pituitary gland contains a little more extractable solid than does that of the ox, whereas horse pituitary gland has almost as much as pig pituitary tissue. Ox pituitary gland is characterized by having a very much smaller 'D' fraction than the other pituitary tissues examined.

DISCUSSION

Relative activities of anterior pituitary tissues from the different species examined.

The results showing the relative hormonal activities of the pituitary glands of the different species are summarized diagrammatically in Fig. 6. It is clear that human pituitary tissue is very much more active gonadotrophically than that of any of the other species examined, but it has a small, though by no means negligible, content of prolactin and of thyrotrophin. Horse pituitary gland is next in order of activity with respect to gonadotrophin as assayed by the rat ovary test, the activity in this respect of the other species examined being very slight. Nevertheless, as determined by the rabbit ovulation test, ox pituitary tissue is as active gonadotrophically as horse pituitary tissue, and pig and sheep pituitary tissues are even more active than that of horse.

The glands from sheep and ox contain the most prolactin, pig and horse tissues containing negligible amounts. Pig pituitary tissue is most active thyrotrophically: ox and sheep tissues both possess a fair degree of activity of this type, but horse tissue is negligibly active in this respect.

These results suggest that for the purpose of preparing gonadotrophic extracts human and horse pituitary tissues are most desirable. Either sheep or ox pituitary tissue is convenient for the preparation of prolactin, while pig and ox tissues are suitable for the preparation of thyrotrophin.

In order to bring out any relationship existing between the amounts of the hormones present in the different pituitary tissues the quotients or products of the figures representing the various types of activity have been examined. The most promising results are given in Table VIII, which shows that for pig, sheep, and ox tissues the quotients

$$\frac{\text{gonadotrophic activity (ovulation test)}}{\text{thyrotrophic activity}}$$

are of the same order, possibly indicating some degree of relationship between the types of activity. The evidence for a relationship between the amounts of thyrotrophic and luteinizing hormone in pituitary tissues from different species is discussed in the introduction. Our results indicate

that the proportion of gonadotrophic activity in human and horse pituitary tissues is very much greater than that in any of the other species examined. For human, sheep, and ox pituitary tissues the quotient

$$\frac{\text{prolactin content}}{\text{thyrotrophin content}}$$

is approximately constant, possibly indicating some degree of proportionality for these species. Finally, the product (thyrotrophic activity)× (gonadotrophic activity assayed by ovarian growth) is of the same order for pig, sheep, and ox tissues, suggesting some slight degree of inverse proportionality for these tissues. It must be confessed that the evidence for the existence of these relationships is not very convincing.

Table VIII. *Possible relationships between the contents of pituitary hormones on the tissues examined*

	Value of expression for:		
Species	$\left[\dfrac{\text{Gonadotrophin}\ (\text{ovulation test})}{\text{Thyrotrophin}}\right]$	$\left[\dfrac{\text{Prolactin}}{\text{Thyrotrophin}}\right]$	$\left[\dfrac{(\text{Thyrotrophin})\times(\text{Gonadotrophin-ovarian growth test})}{10^6}\right]$
Horse	85	6	114
Man	93	26	818
Sheep	25	29	44
Ox	14	22	27
Pig	16	0·8	50

(The hormone contents in the above expressions have been taken in terms of units per 100 g. of dried tissue.)

Methods for the preparation of the various active substances.

On the basis of our results it is not easy to make any generalization about the methods suitable for the extraction of different hormones. For instance, the extraction of thyrotrophin by aqueous alkali, alkaline alcohol, or 50% pyridine is satisfactory with ox, pig, and horse tissues, but not with sheep pituitary tissue. On the other hand, sheep thyrotrophin was extracted satisfactorily by the van Dyke acid buffer. Again, methods for the alkaline extraction of prolactin were satisfactory with tissues from all the species examined with the exception of sheep. Undoubtedly the properties of the active principles will be greatly influenced by the other materials present in crude extracts, and the observation that thyrotrophin in crude extracts of sheep pituitary tissue behaves differently from thyrotrophin in crude extracts of other pituitary tissues cannot be considered as evidence that sheep thyrotrophin differs from thyrotrophin present in pituitary glands of other species.

In general, the method of Bates and Riddle [1935] is very satisfactory for the preparation of prolactin free from thyrotrophin and gonadotrophin,

and is also suitable for the preparation of extracts with thyrotrophic activity. For the preparation of extracts with gonadotrophic activity, simple aqueous alkaline extraction or extraction with the van Dyke acid buffer, seem to be the most satisfactory methods.

<div align="center">SUMMARY</div>

1. The content of thyrotrophin, gonadotrophin, and prolactin of acetone-desiccated anterior pituitary tissue from man, horse, sheep, pig, and ox have been determined. The results, which are summarized in Table I, show that the greatest amounts of thyrotrophin, gonadotrophin, and prolactin are contained in the pituitary tissues from pig, man, and sheep respectively.

2. Gonadotrophic activity, assayed by ability to induce ovulation in the oestrous rabbit, is present to a substantial extent in the pituitary tissue from all the five species examined, but when *gonadotrophic activity is assayed by capacity to cause ovarian growth in the immature rat*, the pituitary glands of man and horse are found to contain large amounts, whereas those of sheep, pig, and ox possess only slight activity.

3. The van Dyke acid-buffer method and the Bates-Riddle alkaline aqueous alcohol method are both satisfactory for the preparation of extracts having thyrotrophic activity. The Bates-Riddle method was also found to be satisfactory for the preparation and purification of prolactin. Aqueous alkaline extraction or extraction with acid buffer (van Dyke) were found to be satisfactory procedures for the preparation of extracts with gonadotrophic activity. Alkaline aqueous alcohol was found to be greatly inferior to simple aqueous alkali for the extraction of gonadotrophic activity (ovarian growth assay) from horse pituitary tissue.

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Methods and Results

A number of 4–5-month-old female rats of the Long-Evans strain, bred in our laboratory, were examined by laparotomy on the 1st day of oestrus. With minor exceptions the uteri of all the animals exhibited complete oestrus, the essentials of which are hypertrophy, hyperaemia, and distension. The ovaries contained either mature follicles or numerous corpora lutea of apparently recent origin. The animals were segregated into three groups and fed on different diets as follows:

(1) A low protein (7%) diet, otherwise complete (the protein being derived from the milk powder present in the diet).

(2) A complete diet containing 4% NH_4Cl.

(3) A complete diet containing 8% $NaHCO_3$.

All the rats lost weight, especially during the first few weeks. The usual weight lost was about 5 g. weekly; but those on the NH_4Cl diet lost as much as 10 g. weekly at first. The rats were smeared daily for about 8 weeks, after which they were autopsied. They were, however, laparotomized before the end of the experiment, and a section of the uterus and one ovary removed for microscopic study.

The changes in the vaginal smears of the rats in the first two groups were quite similar. They exhibited regular vaginal oestrous cycles for about 3 weeks, after which the dioestrous periods lengthened gradually and, after the 5th week, became rather infrequent. The group of rats on $NaHCO_3$ differed from the others in that they continued to have normal vaginal cycles for about 6 weeks. During the 3rd and 4th weeks on the various dietary régimes all rats were opened on the 1st day of vaginal oestrus, and the uteri and ovaries were examined for the completeness of the oestrous responses. It is important to examine the uteri during late pro-oestrus or early oestrus, since the typical uterine oestrous phenomena regress soon after the fully cornified vaginal smear is obtained. The uterine oestrus observed by gross examination in our rats was classified into one of two groups: complete oestrus, in which the uteri were distended and hyperaemic; and incomplete oestrus, where the uteri were pale and not distended, and resembled those in normal dioestrus. Table I summarizes these observations:

Table I. *Type of uterine response observed during vaginal oestrus of rats on various diets*

Diet	Complete oestrus	Incomplete oestrus
Low protein	3	15
NH_4Cl	7	9
$NaHCO_3$	11	4

It can be seen from this table that the completeness of the rats' oestrous

cycles was significantly affected by the first two diets, but not by the NaHCO₃ diet. In some of the uteri labelled 'incomplete' there was considerable atrophy resembling almost that seen in castrate rats. These abnormalities of oestrus could not be detected by the examination of the vaginal smear alone, since in rats with both types of uterine response the vaginal smears were typically oestrous, which is ordinarily regarded as signifying normal ovarian activity.

The sections of uteri removed at the 3rd or 4th week were quite variable in microscopic appearance. Those of rats on the NaHCO₃ diet appeared relatively normal, except for occasional low columnar epithelium of the endometrium. In the other two groups the epithelium was most frequently cuboidal and often atrophic, resembling the involuted epithelium of the castrate. It was difficult to predict, however, from the gross appearance of the uterus, the exact state of the endometrium.

Definite gross changes in the ovaries were also observed. Although the ovaries of the rats with incomplete oestrus appeared normal in size at the 3rd or 4th week, mature follicles were few or absent. When these rats were examined at the 6th or 8th week, the ovaries were definitely atrophic, containing few corpora lutea, and only an occasional mature follicle. It should be noted that in the interval between the 3rd and 8th week, during which such marked ovarian degeneration was in process, vaginal oestrus was observed at least several times in most animals.

Microscopic examination of the ovaries of those rats which demonstrated incomplete oestrus generally exhibited definitely atrophic corpora lutea and an absence of large follicles. There was observed, in addition, considerable thecal and interstitial cell hypertrophy, the cells resembling those which we have already described in rats which were X-irradiated and which also exhibited partial oestrus. These results were also observed by Guilbert and Goss [1932] in their rats on a low protein intake.

Control Experiments forestalling Possible Objections to the above Results and Interpretations

We considered the possibility that under the experimental conditions the uterus might have lost its capacity to respond to the normal ovarian oestrogen and might thus have failed to show a complete oestrus at the time of vaginal cornification. This possibility was dismissed on the following grounds. Rats placed on these diets and showing partial spontaneous oestrus were castrated. Maintaining them on the same diets, they were injected with about 10 I.B.U. of a complete oestrogen, oestradiol benzoate. Typical complete oestrous uteri were obtained in all cases.

A criticism of our previous work on partial oestrus also required further investigation. It had been suggested that partial oestrus might be due

to the elaboration by the ovary of a sub-threshold amount of a single complete oestrogen, sufficient to induce cornification of the vagina, but insufficient to result in a complete uterine reaction [Wintersteiner and Smith, 1938]. This criticism was based mainly on the work of Marrian and Parkes [1930], who demonstrated that 200 times the vaginal requirement of oestrogen in the mouse was necessary to induce uterine oestrus. Others have also shown the presence of a uterine-vaginal threshold difference. Clauberg [1936] claimed that the induction of uterine oestrus in the castrate mouse required 10 times as much oestrogen as the induction of vaginal cornification. Szarka and Kurtz [1938] recently obtained a uterine-vaginal ratio of 5 or 6 to 1, in the rat. Opposed to this, however, is other recent evidence which indicates that the uterus is equally as sensitive as the vagina to oestrogenic substance. Levin and Tyndale [1937], working with the mouse, and Lauson, Heller, Golden, and Severinghaus [1939] with the rat, claim the uterus to be very sensitive in responding to oestrogen elaboration by the ovary, and they use the weight increase of the uteri as the most delicate test of ovarian activity.

The following experiments demonstrate that, in our hands, the uterine-vaginal threshold difference in castrates to injected oestrogen is inadequate to account for the partial oestrous responses which we have obtained:

Rats 3 months old were ovariectomized; 10 days later they received varying doses of oestrone daily for 3 days. Vaginal smears were obtained after 48 and 72 hours. At the end of 72 hours they were sacrificed and the uteri examined, weighed, and sectioned. Table II summarizes the results:

Table II

No. of rats	Oestrone dosage	Body-weight	Uterine weight	Gross uterine response	Vaginal smears at 72 hours
		g.	mg.		
8	None	180	100 (90–110)	0	8 negative
8	15 I.U. oestrone × 3	165	230 (205–254)	8 full oestrus	8 cornified
8	10 I.U. oestrone × 3	180	242 (210–278)	7 full oestrus; 1 moderate oestrus	8 cornified 8 cornified
24	5 I.U. oestrone × 3	180	153 (110–208)	12 slight oestrus; 12 negative	13 negative 11 cornified

It may be seen from Table II that a daily dose of 5 I.U. of oestrone for 3 days was capable of inducing vaginal cornification in about 50% of the rats. This amount also produced definite increase in uterine weight. Twice this amount of oestrogenic substance induced a gross uterine oestrus reaction with hyperaemia, distension, and considerable increase in weight. This effect was somewhat more intense with injections of 15 I.U. daily. The microscopic appearance of the uteri paralleled the macroscopic

observations. The epithelium of the endometrium of rats receiving 5 I.U. daily was cuboidal, that of rats receiving 10 I.U. was low to tall columnar, while tall columnar epithelium was consistently found in those receiving 15 I.U. of oestrone daily. Since 5 I.U. of oestrone induced full vaginal cornification in 50% of our rats, this amount of the hormone may be considered to represent the minimal requirement for vaginal cornification. On the same basis the uterine requirement would be more than once but less than twice the vaginal requirement. But in view of the fact that all of our rats which exhibited incomplete oestrus did have full vaginal oestrus, it is necessary to suppose that their ovaries were secreting at least twice the vaginal requirement of oestrogenic substance. Under these circumstances most of the uteri would also have undergone the complete oestrous reaction. It is, therefore, impossible to account for the results in Table I on the basis of a difference in threshold between the uterus and vagina.

The marked discrepancy between our results on the uterine-vaginal difference in oestrogen response and those of Marrian and Parkes [1930] can be explained by the fact that in their experiments the animals (mice) were castrated at least 6 months previously. The following data reveal that the longer the period following castration the less the uterine response to oestrogen.

Into adult rats of the same age, but which had been castrated at various times previously, we injected about 10 I.B.U. of oestradiol benzoate daily for 3 days. Normal complete uterine oestrus was obtained at the end of 72 hours in rats castrated for as long as 3 months. Those castrated 5 months previously had much feebler responses, the uteri being moderately distended and only mildly hyperaemic. This loss of reactivity of the uterus to oestrogen after long periods of castration was further demonstrated by injecting a similar series of rats for 20 days with about 10 I.B.U. oestradiol benzoate daily. The following table illustrates quantitatively the poorer development of the uteri of rats which were castrated at earlier dates.

Table III

No. of rats of the same age	Interval following castration	Average weight of uteri after oestrogen treatment
		mg.
14	1 week	420
12	3 months	365
12	5 months	310

DISCUSSION

Our results show that adult female rats on unbalanced diets may suffer varying degrees of ovarian atrophy and continuous uterine dioestrus, while at the same time exhibiting cyclic cornification of the vagina. Thus

the adequacy of experimental diets as regards normal sex function cannot be judged solely on the basis of the vaginal smear technique. This supports our previous conclusions, derived from experiments under other conditions, that vaginal cornification is not a reliable index of the state of the uterus or of normal ovarian function. For the latter purpose, gross and microscopic examinations of the uterus and ovaries are essential. These considerations seem to be both timely and important, in view of the wide application which is being made of the vaginal smear technique for assay purposes in animals, and because of the recent attempts to introduce this method for diagnostic purposes in humans.

The mechanism through which our diets induced partial oestrous reactions is obscure. It has been shown, however, that the incomplete oestrus is not due to any inherent uterine deficiency, but depends upon damage to the ovaries. Whether the latter results from changes in the secretion of the gonadotropic hormones of the anterior hypophysis, or is a direct result of the dietary deficiency upon the ovaries, is still problematic [Mulinos, Pomerantz, Smelser, and Kurzrok, 1939]. The possibility that the damaged ovaries maintain a normal oestrogenic secretion, but in amounts so small that the vagina responds while the uterus does not, has been investigated and found to be untenable. This conclusion is also supported by the work of others with other species of animal. Roentgen irradiation of the ovaries in ferrets, for example, may result in an oestrus in which the uterus is swollen and hyperaemic, but in which there are no vulvar manifestations [Parkes, Rowlands, and Brambell, 1932]. On the other hand, the administration of A.P.L. to the hypophysectomized ferret induces vulvar congestion without any significant oestrous changes in the uterus [McPhail, 1933]. It would be difficult to account for these opposite oestrogenic effects on the basis of a threshold difference in sensitivity to oestrogen.

Our previous work leads us to conclude that our present results are due to the failure of the damaged ovaries to secrete one or more of the various oestrogenic factors, which together are responsible for the composite effect which we recognize as the complete oestrous reaction. In this connexion we have previously presented evidence that the theca cells elaborate an oestrogen which affects the myometrium, while the secretion of the granulosa stimulates endometrial development [Freed and Soskin, 1937]. Consistent with this, the ovaries of the rats affected by diet in the present work exhibited atrophic corpora lutea and an absence of large follicles, but considerable theca and interstitial cell hypertrophy.

The possible application of our work to the understanding of gynaecological disturbances in the human female can only be conjectured at present. It may be pointed out, however, that an excess of a single

oestrogen can hardly account for the occurrence of endometrial hyperplasia and of myometrial hypertrophy (fibroids), independently of each other. It is possible, therefore, that these conditions might occur as the result of a disturbed relationship between the various oestrogenic factors secreted by the ovaries. It is also possible that conditions as simple and as frequent as an unbalanced diet might play a role in various gynaecological disorders.

SUMMARY

The vaginal mucosa in the rat is not an accurate indicator of complete ovarian function. Under certain dietary conditions normal rats develop vaginal oestrus in the presence of uterine dioestrus and ovarian atrophy. We have explained the mechanism of such partial oestrous phenomena by postulating that the rat's ovary elaborates several distinct factors, or oestrogens. The effects of these partial oestrogens differ qualitatively, and the complete oestrous phenomenon is a composite of the several partial effects.

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STUDIES ON THE RELATIONSHIP OF VITAMIN E (TOCOPHEROLS) TO THE ENDOCRINE SYSTEM

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ALTHOUGH it is now established that deprivation of vitamin E may lead to disturbances of structure and function of many tissues other than those primarily concerned in the reproductive cycle, it is not surprising, when the striking nature of the testicular degeneration and the curious character of the typical resorption in the female are borne in mind, that there has been a tendency to concentrate attention on the question whether the vitamin plays an essential part in the reproductive cycle.

The most direct approach has been made by investigating whether the vitamin itself exerts a gonadotrophic action. Up to the present the evidence has been inconclusive. The most striking claim is that of Szarka [1929], who stated that oral or parenteral administration of vitamin E concentrates produces oestrus in immature female rats. Later, Verzár [1931] recorded that injection of similar materials produced hypertrophy of the uterus in similar animals, but not after ovariectomy. Diakov and Krizenecky [1933 a] as well as Lesbouyrie, Berthelon, and Bajé [1937] were, however, unable to confirm Szarka's and Verzár's findings. Recently, Underhill [1939] has supported the view that vitamin E preparations exert gonadotrophic activity when injected into immature female mice, whereas Demole [1939] did not observe any effect on the reproductive system when doses of α-tocopherol as large as 0·5 g. per kg. were given.

It will be recalled that Verzár's belief was that vitamin E is concerned in the formation of one or more of the hormones of the anterior pituitary [Verzár, 1931; Verzár, Arvay, and Kokas, 1931; Verzár and Kokas, 1931]. The suggestion that pituitary function is depressed in the condition of vitamin E deprivation was rejected by Evans [1932] because parenteral administration of fresh hypophyseal substance failed to prevent the typical resorption in E-deficient females and also because the striking contrast between the severity of the degeneration of testicular structure and the maintenance of normal ovarian structure was incompatible with the accepted views of pituitary function.

The pituitary came into the picture again, however, when Nelson [1933] found that testicular degeneration in the condition of E-deficiency is

associated both with histological changes and with an increased amount of gonadotrophic hormones in the anterior lobe. Gierhake [1933] also made similar observations. These 'castration changes' were marked in the male pituitary following a period of E-deficiency, but Nelson did not detect them in female rats. Later, McQueen-Williams [1934] claimed that pituitaries removed from females fed on E-deficient diets for 13–20 months and which had also had resorption-gestations, exerted more than twice the gonadotrophic action shown by glands removed from adequately nourished females. This appeared to indicate that E-deprivation at length produces gonadal impairment in the female as well as in the male.

The view that the picture of E-deficiency might be clarified by studying what appeared to be disturbances of the hormonal activity of the pituitary was strengthened by the observation of Rowlands and Singer [1936] that the luteinizing potency of the glands, as measured by the rabbit-ovulation test of Hill, Parkes, and White [1934], is significantly reduced in that condition and also by the work of Barrie [1937 a, 1937 b, 1937 c].

In the following experiments an attempt has been made to determine what possible relationship the endocrine organs might play in the changes found in E-deficient male and female rats.

METHODS

The E-deficient rats used in these experiments were ones which had been placed at weaning on one or other of two E-deficient diets, given below.

U.C. diet		Vitamins laboratory diet	
'Glaxo' casein (ashless E) . . . 18		Casein . . . . . 18 parts
Rice starch 45		Rice starch 50
Lard 12		Lard 20
Yeastrol (yeast extract) . . 8		Dried brewer's yeast . . 14
Cane sugar 10		Salt mixture 5
'Glaxo' salt mixture . . . 5		Daily cod liver oil
Cod liver oil 2		

The animals were weighed at regular intervals and maintained on the diet for various lengths of time. The normal stock from which immature females or others used for hypophysectomy were taken were fed on a normal diet of the same composition as described by Thomson [1936]. Other control animals were fed on the balanced rat ration compounded by Messrs Agricultural Food Products Limited, London, W. 6.

At autopsy the various endocrine organs were dissected and fixed in Bouin's fluid. In some cases they were weighed after partial dehydration in 70% alcohol; this is noted in the tables. The gonads were examined histologically.

In some experiments fresh pituitary glands were used for implantation;

in others they were first dehydrated by placing in acetone and then dried. Suspensions made by grinding the dried glands in water at a pH of 9–10 were kept in the ice-chest until required. An extract of the serum of pregnant mares 'Antostab' (Boots) and an extract of the urine of pregnant women 'Physostab' (Boots) have been employed to produce stimulation of the gonads. In replacement therapy with vitamin E, either concentrates prepared by the method described by Moss and Drummond [1938] or synthetic α-tocopherol acetate generously supplied by Roche Products Limited have been used.

RESULTS

Effects of Vitamin E on Normal Rats

Immature females. A group of 5 immature rats (40–45 g.) were given daily subcutaneous injections of 5 mg. of synthetic α-tocopherol acetate (as a 20% solution in olive oil) for 5 days and killed on the following day. Another group of 5 animals were given orally the same dose of tocopherol. At autopsy it was found that in all cases the vagina was closed and that the ovaries and uteri had remained in the infantile condition.

Hypophysectomized adult females. Vitamin E concentrate (73% tocopherol) equivalent to 25 mg. vitamin E was given by mouth to adult female rats, from 10 to 18 days after removal of the pituitary gland. The vaginal smear did not alter from the dioestrous condition and no effect of the vitamin E could be demonstrated on the body-weight or weights of the ovaries or adrenal glands.

Gonadotrophic Hormone Content of Pituitaries from E-deficient Rats

In the first experiment immature female rats were injected subcutaneously with the equivalent of one pituitary daily for 5 days and killed on the following day. The E-deficient rats from which the pituitaries were obtained had been fed on a deficient diet for about 9 months. The results may be seen in Table I.

Table I. *Assay of pituitaries from E-deficient rats on immature female rats.*
(Dose = to 1 gland daily for 5 days)

Source of pituitary gland	Sex	Body-wt. g.	Uterus mg.	Ovaries mg.	Vagina
E-deficient	M	60	73	63	open
Normal	M	52	73	42	open
Normal	M	56	100	43	open
E-deficient	F	53	50	7*	closed
Normal	F	50	41	9	closed
Normal	F	56	73	11	open

* The weights of the ovaries in all groups of this experiment were definitely smaller than are normal for this range of body-weight.

These results confirmed Nelson's observations, but not those of McQueen-Williams; the low hormone content of the pituitaries in the case of the female rats, however, made the ovarian response of doubtful significance.

In a further experiment immature female rats (40–45 g.), hypophysectomized for 7–10 days, were injected with the pituitary gland suspensions of female rats, E-deficient for various periods. These results are shown in Table II.

Table II. *Assay of pituitaries from E-deficient female rats on immature hypophysectomized female rats*

Source of pituitary gland	Total dose. = no. of glands	Injections days	Uterus mg.	Ovaries mg.
—	0-control	—	18	8
—	0-control	—	25	7
Normal rats	5	5	215	14
„ „	5	5	220	14
E-deficient for 3 months	4	4	120	15
„ „	5	5	85	20
„ „	5	5	255	25
„ „	5	5	226	26
Normal rats	20	10	255	137
E-deficient for 3 months	20	10	200	84
E-deficient for 12 months	20	10	226	42

These results are conflicting in that the animals receiving the equivalent of 5 glands gave a different response from those injected with 20 glands. In the former series the E-deficient pituitaries contained more gonadotrophic hormone than those from normally fed rats. When the larger dosage was used the hormone content of the glands diminished as the severity of the E-deprivation increased. Histological examination of the ovaries of the rats receiving the larger dose showed that those stimulated by the pituitaries of normal rats contained numerous large follicles and corpora lutea. The pituitaries of rats E-deficient for 12 months, on the other hand, caused only follicular growth. This may be interpreted as an indication of an alteration of the gonadotrophic hormone content of the pituitary gland in E-deficiency. When experiments of a similar nature were repeated, using the pituitaries from long E-deficient male rats, it was found that a stimulation of both follicles and corpora lutea occurred.

Treatment of E-deficient Female Rats

Endocrine gland weights. It was considered of interest to collect the data concerning the weights of the endocrine glands of E-deficient female rats to see if they differed appreciably from those of control animals. Groups

of 20 or 30 animals have been used for comparison and the weights obtained are listed in Table III.

Table III. *Endocrine gland weights in E-deficient female rats*

	Normal rats	E-deficient 3–6 months virgin	E-deficient 9–12 months virgin	E-deficient 9–12 months 1 or more resorptions
Body-weight g. .	154·8	141·5	199·0	207·0
Uterus mg. .	365·3	304·4	—	763·3
„ mg./100 g. .	238·22 (S.D. 92·2)	216·5 (S.D. 112·5)	—	369·7 (S.D. 136·26)
Ovaries mg. .	52·7	47·9	74·0	88·9
„ mg./100 g. .	33·65 (S.D. 7·25)	32·84 (S.D. 4·16)	38·23 (S.D. 9·28)	43·74 (S.D. 7·84)
Pituitary mg. .	8·3	7·6	11·5	12·6
„ mg./100 g. .	5·38 (S.D. 0·73)	5·42 (S.D. 0·78)	5·86 (S.D. 1·36)	6·15 (S.D. 0·67)
Adrenals mg. .	52·6	54·9	55·25	56·6
„ mg./100 g. .	33·69 (S.D. 4·42)	39·25 (S.D. 8·00)	28·24 (S.D. 5·5)	27·77 (S.D. 2·29)

With the exception of those for the adrenal glands, these figures do not show any significant differences between the E-deficient and the normal control rats. The smaller weights of the adrenals per 100 g. of body-weight seen in the cases of E-deficiency are statistically significant, but we do not relate them to the dietary defect, since similar figures were obtained in examining a large group of female rats which had been fed for many months on a diet exceptionally rich in wheat germ oil of proven potency.

Response to Antostab. The response to an extract of pregnant mare serum (Antostab) of the ovaries of female rats after 4–6 months on an E-deficient diet has been determined. Groups of 5 or 10 animals received injections of 5 m.u. daily for 5 days followed by 20 m.u. for 5 days, and were killed on the day after the last injection. The results are shown in Table IV.

Table IV. *Effects of Antostab on E-deficient female rats*

		Average per 100 g. body-weight	
Diet	Treatment	Uterus mg.	Ovaries mg.
Normal	None	238	33
Normal	Antostab	285	79
E-deficient	None	216	32
E-deficient	Antostab	290	60

On sectioning the ovaries showed well-developed follicles and corpora lutea similar to those produced in the control injected rats.

Attempts to prevent resorption. Various attempts were made to alter the hormone balance in E-deficient rats which had become pregnant in order to see if it was possible to prevent the resorption which regularly occurs. The following substances were administered to E-deficient rats as soon as pregnancy was established:

(*a*) Injection of saline suspension of dried rat pituitary glands equivalent to 5 mg. of fresh gland daily.

(*b*) Implantation of two fresh rat pituitary glands every second day.

T

    (c) Daily injections of 10 m.u. Antostab (extract of pregnant mare serum).

    (d) Daily injections of 5 m.u. Physostab (extract of human pregnancy urine).

    (e) The subcutaneous implantation of 5 mg. tablet of progesterone.

    (f) Implantation of 20 mg. tablet of testosterone propionate.

None of these forms of treatment was found to be effective in preventing foetal resorption.

Treatment of E-deficient Male Rats

Endocrine gland weights. The weights of the various endocrine glands have been compared in normal rats with those of E-deficient male rats. Groups of 20 or 30 animals were used. These results are shown in Table V.

Table V. *Endocrine gland weights in E-deficient male rats (9–10 months deficiency)*

	Normal rats	E-deficient
Body-weight g.	231	236
Testes mg.	2414	810
„ mg./100 g.	1060·8 (S.D. 213·8)	341·7 (S.D. 67·7)
Prostate mg.	350	230
„ mg./100 g.	142·7 (S.D. 81·44)	102·7 (S.D. 47·7)
S. vesicles mg.	266	95·2
„ mg./100 g.	68·84 (S.D. 4·16)	46·6 (S.D. 8·99)
Pituitary mg.	7·5	8·5
„ mg./100 g.	3·19 (S.D. 0·6)	3·61 (S.D. 0·33)
Adrenals mg.	35	32
„ mg./100 g.	16·2 (S.D. 3·54)	15·7 (S.D. 4·41)

These figures show the striking alteration in weight which occurs in the testes after E-deprivation. The prostate and seminal vesicles, on the other hand, are relatively slightly affected.

Response to treatment. Attempts have been made to restore the testes of E-deficient rats by treatment with gonadotrophic extracts, either alone or supplemented by vitamin E by mouth. Animals on a deficient diet for about 6 months were treated daily for 14 days, and the results are shown in Table VI.

No evidence of testicular regeneration from the weights or from microscopic examination was found. There was, however, evidence of increased activity of the interstitial tissue under the stimulus of the Antostab. Experiments of a similar type in which administration of pregnant mare serum extract (Antostab), together with vitamin E concentrates, was supplemented by doses of 5 m.u. and 10 m.u. of Physostab (human pregnancy urine extract) daily for 14 days also failed to produce any detectable restoration of spermatogenesis.

In another experiment male rats which had been on the deficient diet about 150 days were fed for 26 days on a diet very rich in E. It contained

12% of a potent wheat germ oil and was supplemented with a daily dose of concentrate providing about 5 mg. of the vitamin. After this period they were injected with 20 m.u. daily of Antostab for 24 days, the diet being maintained. They were mated with a succession of normal females during this period, but no pregnancies resulted and the males appeared to have diminished sex interest. At autopsy the testicular weights were still within the range found at this stage of atrophy caused

Table VI. *Effects of Antostab and vitamin E on E-deficient male rats.*
(5–6 months deficient)

Treatment	Body-weight g.	Testes g.	Testes g. per 100 gm.	Seminal vesicles g.	Prostate g.	Pituitary mg.	Adrenals mg.
Nil	260	2·86*	1·1	0·93	0·80	6	34
	287	3·12	1·08	0·48	0·42	7	47
	238	2·50	1·09	0·47	0·36	7	34
5 mg. E daily	250	1·97	0·79	0·26	0·22	7	44
	198	1·98	1·00	0·38	0·51	6	39
	160	1·53	0·95	0·27	0·34	7	34
5 mg. E+10 m.u. Antostab daily	240	2·93	1·23	1·74	1·15	5	32
	300	2·93	0·97	1·35	1·05	8	44
	240	2·17	0·90	0·85	0·79	6	31
10 m.u. Antostab daily	230	2·30	1·0	1·08	0·77	5·5	38
	225	1·86	0·82	1·61	0·97	6	38

* It will be noted that the weights of the testes of the untreated animals is about the same as those given for normal animals in Table V. This is mainly due to the fact that the rats had been fed on the deficient diet for only from 5 to 6 months. We have encountered considerable variations in the extent to which atrophy occurs in the first stages of E-deficiency, e.g. another group which had been fed on the deficient diet for 6 months had testes averaging only 1 g. The animals to which the figures in Table VI refer were, however, drawn from one group and are, therefore, presumably comparable.

by E-deprivation, and the microscopic study failed to reveal any signs of regeneration of the spermatogenic tissues.

In order to study the response of the interstitial cells of the testes of E-deficient rats to stimulation by gonadotrophic hormones it was considered essential first to remove the animals' pituitary glands. A number of male rats which had been on E-deficient diets for 12 months were hypophysectomized and then injected with Antostab. The results are given in Table VII.

Table VII. *Effects of Antostab on hypophysectomized E-deficient male rats.*

No. of rats	Condition	Treatment	Testes mg.	Seminal vesicles mg.	Prostate mg.	Adrenals mg.
3	Normal	None	1096	857	893	41
3	Hypophysectomized 10–12 days	None	518	218	325	33
2	Hypophysectomized 10–13 days	10 m.u. Antostab daily	762	1761	1065	26

It may be seen that in this series of rats the seminal vesicles and prostates were of a normal size in the E-deficient animals despite the atrophic condition of the testes. The interstitial cells appear, therefore, to be able to maintain a normal secretion of androgens, whereas the spermatogenic tissues atrophy and become functionless. The microscopic appearance of the testes of one of these animals may be seen in Plate I, Fig. 1. After hypophysectomy the size of the testes is markedly reduced, and the interstitial cells cease to function, as indicated by the atrophic condition of the accessory organs. A section of testis showing this increased atrophy is reproduced in Plate I, Fig. 2. Pregnant mare serum extract, however, tends to maintain the weight of the testes and stimulates the interstitial cells to liberate androgens, so that a marked increase in size of the prostate and seminal vesicles ensued. Since the spermatogenic tissue of the tubules was unaffected, the testes of the hypophysectomized E-deficient male rat would appear to respond to pregnant mare serum only by interstitial cell stimulation. The microscopic picture may be seen in Plate II, Fig. 3.

DISCUSSION

The experimental results which have been obtained do not indicate that the endocrine system is primarily responsible for the changes observed after feeding rats on an E-deficient diet. From a consideration of the changes observed at autopsy in deficient rats it is apparent that the most striking difference from normal is in the testes of the male animals. Whereas the testes are reduced in weight, the accessory organs were only slightly subnormal. Kudrjaschov [1930, 1931, 1936] has suggested that the effects on the accessories are related to the retardation of growth and general debility of the rats when maintained on deficient diets for prolonged periods. Despite what appears to be a relatively normal production of androgens by the interstitial cells of the testes of E-deficient rats, it has been noted that mating seldom occurs and that the animals exhibit in the later stages a marked lack of sexual interest. This observation was originally made by Evans and Burr [1927] and has been confirmed more recently by Wiesner and Bacharach [1937].

The gonadotrophic hormone content of the pituitary gland of E-deficient female rats would appear to differ only slightly from that of glands of normal rats. The hormone content of the pituitary from E-deficient male rats appeared, however, to be increased when compared with normal glands, as originally shown by Nelson [1933]. When hypophysectomized rats were used as test animals it was found that when the dosage of pituitary gland administered was such that only slight ovarian growth took place the E-deficient rats' pituitaries caused greater stimulation than those from normal rats, an observation that seems to confirm that of

McQueen-Williams [1934]. When the dosage was increased it was noted that the gonadotrophic hormone content of the pituitaries decreased as the length of E-deprivation increased. Also, the luteinizing activity of the pituitaries appeared to diminish, as previously noted by Rowlands and Singer [1936]. An alteration in the relative amounts of follicle and luteinizing hormone content in the pituitary might explain the difference in the results obtained using small and large doses. When the luteinizing activity of the extract becomes reduced the stimulated ovaries are small and without corpora lutea.

Vitamin E, when administered either orally or by injection, to immature rats or to hypophysectomized adult females, was devoid of any activity on the gonads, vagina, or pituitary gland. Claims that vitamin E possesses oestrogenic activity [Szarka, 1929] or gonadotrophic activity [Verzár, 1931, Underhill, 1939] were not confirmed in the experiments recorded.

When female rats deprived of vitamin E were treated with pregnant mare serum extract it was found that the ovarian response was essentially normal. Attempts made to prevent resorption in pregnant E-deficient rats by the administration of various extracts and hormones were, however, all unsuccessful. The observations of Evans [1932] that implantation of fresh pituitary tissue, and of Diakov and Krizenecky [1933 b] that injections of Prolan A, do not enable pregnant E-deficient females to produce living litters have been confirmed. Male E-deficient rats were found to respond to pregnant mare serum extract by a stimulation of the interstitial cells of the testes. Whereas neither vitamin E nor what appeared to be appropriate forms of hormone therapy were found to be effective in preventing tubular degeneration, the interstitial cells were readily stimulated even in hypophysectomized E-deficient animals.

From a consideration of the results obtained it appeared very unlikely that the effects of E-deprivation could be explained by an alteration in function of the anterior pituitary gland, the slight changes recorded being in all probability related to a secondary effect on the pituitary. Shute [1935 a, 1935 b, 1936, 1937] has advocated an alternative theory that deprivation of vitamin E brings about a disturbance in the balance of the hormones which regulate the gonads and sexual cycle. On evidence which appears to us inadequate he has expressed the opinion that the blood of rats suffering from E-deprivation, or of women in certain conditions believed to be due to lack of E, contains an excess of an oestrogen. A failure to confirm the experimental work on which this claim is based forms the subject of another communication [Cuthbertson and Drummond, 1939], but meanwhile, it is of interest to consider how far such an hypothesis is compatible with established facts.

The dominant influence of an oestrogen might be held responsible for

the termination of at least one pregnancy and it might also provide what at first sight would appear to be a fairly satisfactory explanation of the significant retardation of the later stages of growth and the testicular degeneration in male rats seen in E-deficiency [Noble, 1938]. Other evidence is, however, much too strong to permit the unqualified acceptance of Shute's view. An excess of an oestrogen in the circulating blood, presumably becoming more marked as the condition of E-deficiency is prolonged, would not permit a long series of pregnancies, each apparently quite normal in its earlier stages, such as is shown by the E-deficient rat. A relatively small amount of an oestrogen is required to prevent implantation in the rat, but large doses do not disturb an established pregnancy [Parkes, Dodds, and Noble, 1938]. Moreover, an excess of oestrogen would inevitably cause disturbances of the oestrous cycle, such as are not seen in E-deficient female rats. Also, the weight of the ovaries is not decreased and that of the adrenals or pituitaries not increased as would be expected. Finally, it is quite clear that the character of the testicular degeneration in E-deficiency, as shown in Plate I, Fig. 1, which as Mason [1933] and others have emphasized is quite unique, differs materially from that seen after prolonged administration of oestrogens (Plate II, Fig. 4) and also, it should be noted, from that observed in the normal rat after hypophysectomy (Plate II, Fig. 5).

Perhaps the most striking fact about the testicular degeneration in E-deficiency is its irreparable character. And a long series of failures to induce regeneration of spermatogenesis is recorded in the literature (see review by Mason [1939]). The more one considers the condition of the male rat suffering from prolonged E-deficiency, the more does it appear that the primary lesions are in the spermatogenic cells. Several years ago Mason [1933] suggested that vitamin E may be primarily concerned with maintaining nuclear activity and, perhaps, chromatin formation. His view is compatible with the striking differences between the male and the female animal suffering from E-deficiency, and it also provides a more convincing explanation of the failure of the female to carry pregnancies to a successful termination than does any theory we have considered in which a disturbance of hormone balance produced by a lack of vitamin E is held as being primarily responsible for the changes associated with the vitamin deficiency.

SUMMARY

Vitamin E when administered to immature female rats or to hypophysectomized adult female rats did not exert any effect on the ovaries, uterus, or vagina.

The pituitary glands of E-deficient male rats were found to contain an

increased amount of gonadotrophic hormone. Glands from E-deficient female rats showed a decreased amount of luteinizing hormone when tested on immature hypophysectomized rats.

The ovaries of E-deficient female rats responded normally to extracts of pregnant mare serum. In male E-deficient rats such extracts produced only stimulation of the interstitial cells of the testes, and did not affect the degenerated tubules. Hypophysectomy of male E-deficient animals was followed by a further decline in weight of the testes. In such animals pregnant mare serum stimulated only the interstitial elements of the testes.

The suggestion that the effects of vitamin E deficiency are produced by a hormonal imbalance is not supported by the experimental evidence described.

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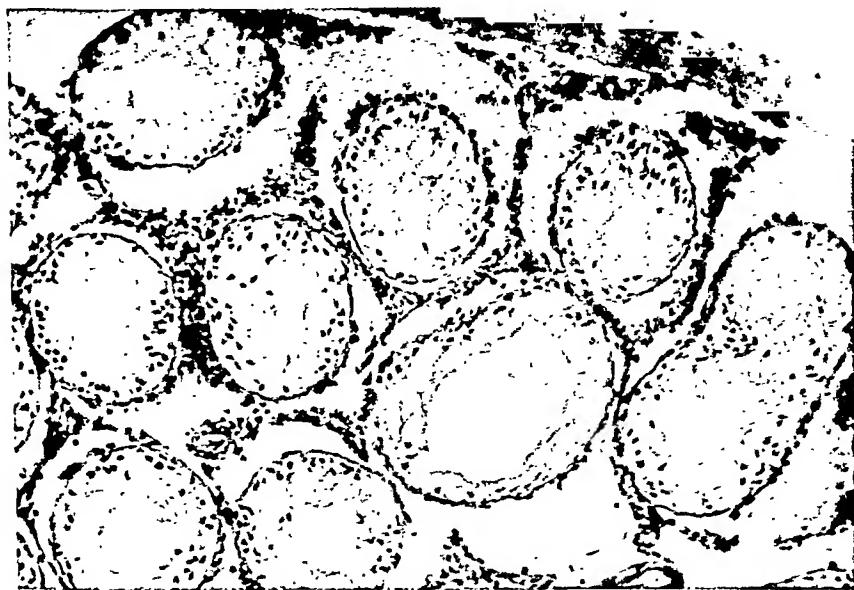
Fig. 1. Section of testis of E-deficient rat after 12 months on diet. Testis removed at operation for hypophysectomy. × 120.
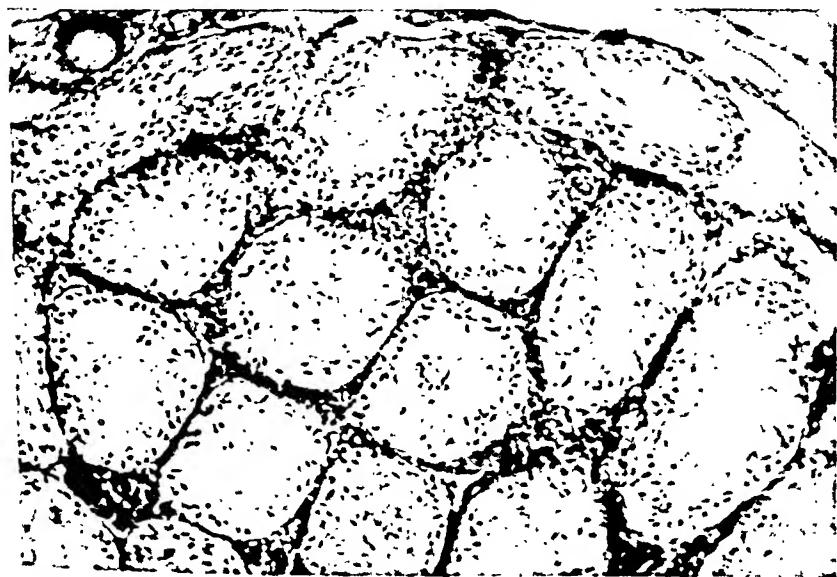
Fig. 2. Control testis of E-deficient rat 9 days after hypophysectomy. × 120.

FIG. 3. E-deficient rat testis 6 days after hypophysectomy, but after treatment with 10 m.u. Antostab for this period. × 120.
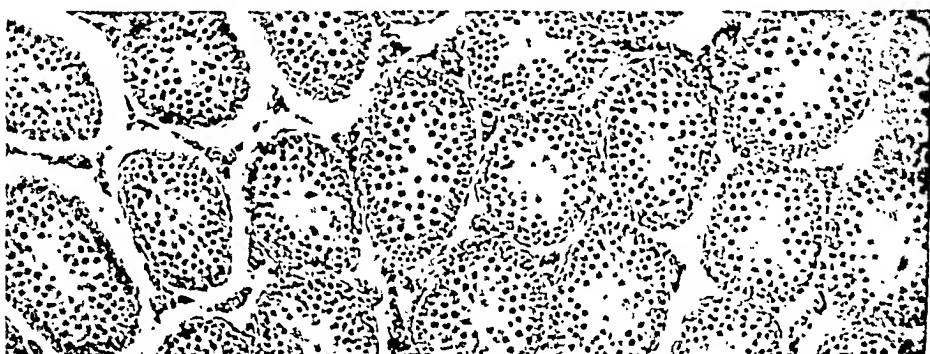
FIG. 4. Rat testicle 19 weeks after subcutaneous implantation of oestrogen crystals. × 120.
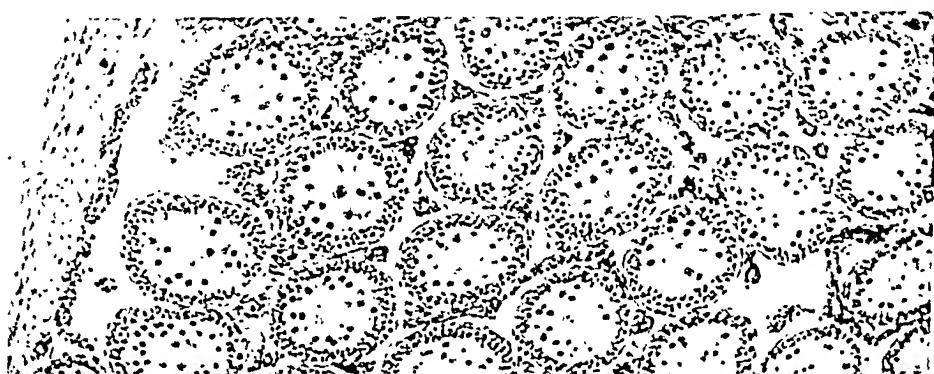
EFFECT OF SUBCUTANEOUS IMPLANTATION OF ADRENALIN TABLETS ON BLOOD-SUGAR AND MILK COMPOSITION IN LACTATING RUMINANTS

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A VERY prolonged effect of certain hormones can be obtained by the subcutaneous implantation of a compressed tablet of undiluted, crystalline substance [Deanesly and Parkes, 1937]. This technique has proved to be specially useful with oestrone and testosterone, or testosterone propionate, and effects lasting many months can be obtained from a single administration. The great efficiency of the method seems to be due to slow and even absorption over a long period, so that the alternation of wasteful excess and sub-threshold concentrations, such as is often produced by the daily injection of solutions, is avoided. In considering whether such a technique could be usefully applied to other hormones it was reasonable to take account of adrenalin, the most rapidly acting and quickly destroyed of the hormones. Further, of the various physiological responses to adrenalin, that of the blood-sugar seemed the most promising for investigation of the effects of prolonged action. It is well known that a single intravenous injection of adrenalin produces only a small and temporary increase in blood-sugar as compared with that which can be obtained by the continuous intravenous perfusion of the same total amount [Cori, Cori, and Buchwald, 1930 *a*]. Subcutaneous administration, admitting of slower absorption and more prolonged action, results in a considerable increase in the blood-sugar (and lactic acid) which may last for several hours [Cori and Cori, 1929]. The possibility of further retarding the absorption of subcutaneously administered adrenalin has been investigated by Kohn and Bulger [1937], who found that the addition of small amounts of zinc sulphate to the solution decreased the maximum blood-sugar resulting from the injection, but increased the time over which an increase could be detected. With a larger quantity of zinc sulphate, absorption was so delayed that little effect was observed.

In view of these results we have investigated the possibility of administering large amounts of adrenalin in the form of a compressed tablet of the pure substance inserted subcutaneously, in the hope that an effective supply would be absorbed from such a depot, not merely for a few hours,

but for several days. About a year after the experiments recorded in this paper began, Keeney, Pierce, and Gay [1939] reported prolonged hyperglycaemia (8 or 9 hours duration) in humans following injections of suspensions of adrenalin in oil.

The possibility of maintaining, by implantation of the solid hormone, a high blood-sugar over a prolonged period suggested an extension of the experiment. It is known that in lactating cows a low blood-sugar over a long period is associated with a decrease both in the amount and the lactose content of the milk [Gowen and Tobey, 1931] and, conversely, it has often been assumed that during a long period of raised blood-sugar the yield and the lactose content of the milk would rise. As yet, however, a satisfactory method of raising the blood-sugar in farm animals over a long period does not seem to have been evolved. The temporary increase in blood-sugar by oral or intravenous administration of glucose to cows sometimes causes a very temporary increase in milk lactose, but has not been shown to increase the milk yield [Whitnah, Riddell, and Hodgson, 1933; see also Nitzescu's [1925] work on the goat]. Indeed, Brown, Petersen, and Gortner [1936] found that intravenous injections of sugars into lactating cows caused hypoglycaemia possibly due to increased secretion of insulin. These authors concluded that intravenous injections of sugars were of doubtful value in studies of lactose synthesis. It seemed, therefore, that while experiments were being carried out on massive dosage with adrenalin in tablet form, it would be useful to use lactating farm animals in which both the yield and composition of the milk could be determined simultaneously with blood-sugar changes. Experiments have, therefore, been carried out on lactating goats and one cow.

METHODS

Adrenalin was used in the form of the free base (B.D.H. Adrenaline). The physical properties of this powder seemed at first to be quite unsuited to the preparation of tablets of sufficient size and solidity. It was found, however, that jamming of the pellet press by the powder could be prevented by coating the die with a thin film of oil; in this way hard, polished tablets, up to 200 mg. in weight, were produced from a standard hand pellet press. Implantation into the animals was carried out under local anaesthesia (Percaine—5 c.c. of a 1/1000 solution intradermally and subcutaneously) by making a small incision through the skin, the wound being closed with two stitches. Doses up to 496 mg. of adrenalin were administered at one time by this method. Contrary to expectations, there was no local reaction, and despite the probable occurrence of local vasoconstriction the material was absorbed in the course of time without

any complications except in one case in which there was sloughing from a small area.

In the experiments on goats, arterial blood samples were obtained from exteriorized carotid loops. In the cow, arterial and venous blood samples were taken approximately simultaneously from the internal iliac artery and the mammary vein by the method of Graham, Kay, and McIntosh [1936]. In all experiments save one, a series of samples was obtained without difficulty from the carotid loops, but only three successive samples could be obtained from the iliac artery (see below). Since fasting blood-sugar values could not be measured because (*a*) of the difficulty of emptying the alimentary tract of a ruminant, and (*b*) starvation would interfere with lactation, blood samples were, as far as possible, taken at the same time of day throughout any one experiment, except that in some experiments an additional sample was taken shortly after the tablet was implanted. Determinations made during the control periods show that in our animals the blood-sugar values so determined varied very little from day to day.

All animals were milked twice daily, at 8.0 a.m. and 3.30 p.m.

Blood-sugar in experiments 1, 3, 4, and 5, and milk lactose, fat, and non-fatty solids, were estimated by the various methods listed by Folley and Young [1938]. In experiments 2, 6, and 7 blood-sugar was determined in tungstic acid filtrates by what was essentially the Hagedorn–Jensen method. Since it is well known that blood lactic acid is increased by adrenalin treatment, and since recent evidence suggests that lactic acid may be a precursor of lactose (see below), blood lactic acid was determined in experiments 4 and 5 by the method of Avery and Hastings [1931] and in experiments 2, 6, and 7 by Lohmann's [1928] method.

RESULTS

Experiment 1. Experiment 1 was carried out on goat Ar. which had been 6 months in milk and weighed 90 lb. The milk yield was approximately constant in the period preceding the experiment at about 27 oz. daily. Observations were made on blood-sugar, lactose (morning milk only), fat, and non-fatty solids, and milk yield. A tablet of adrenalin weighing 48·6 mg. was implanted at 12.30 p.m. on 6 January 1938. The observations showed a sharp rise in blood-sugar from the control level of 62–5 mg. %, so that by 3.30 p.m. on 7 January nearly 100 mg. % was found (Fig. 1). On the following day, 46 hours after implantation of the tablet, the value, 83 mg. %, was still high. At 68 hours after implantation the blood-sugar value was nearly down to normal. The lactose percentage in the milk showed a definite but temporary rise corresponding to that of the blood-sugar. The milk yield was steady for 2 days after the

implantation, but was afterwards irregular with a tendency to fall. The results indicate that there was no significant change in the daily fat percentage, but that the non-fatty solids showed a slight rise after the implantation.
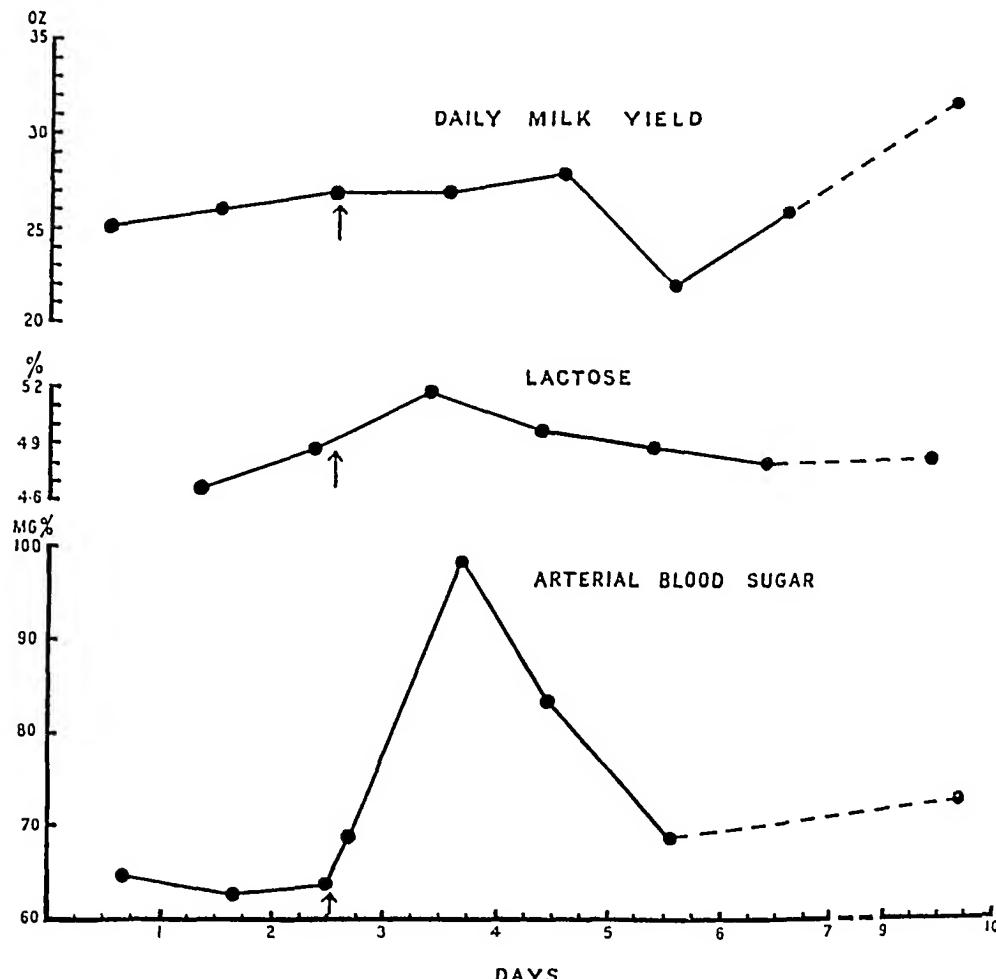
Fig. 1. Goat Ar. (experiment 1). Adrenalin tablet implanted at ↑.
Note.—In all figures the following conventions have been adopted:
(*a*) Each day begins and ends at midnight indicated by the numerals.
(*b*) The results of blood analyses are shown at the hours at which the samples were taken.
(*c*) Except where otherwise stated, daily milk yields are entered at noon, i.e. midway between the a.m. and p.m. milkings.

Experiment 2. Experiment 2 was carried out on goat Ar. when $10\frac{1}{2}$ months in milk. The daily milk yield was rising slowly at the time of the experiment. Lactose, fat, and non-fatty solids were determined in composite samples made each day by combining samples of milk obtained that morning and the previous evening in proportion to the yields. Blood-sugar and lactic acid were also estimated at suitable intervals, except that on

the second day after adrenalin administration it was unfortunately not possible to obtain a blood sample. After a control period of 6 days, a
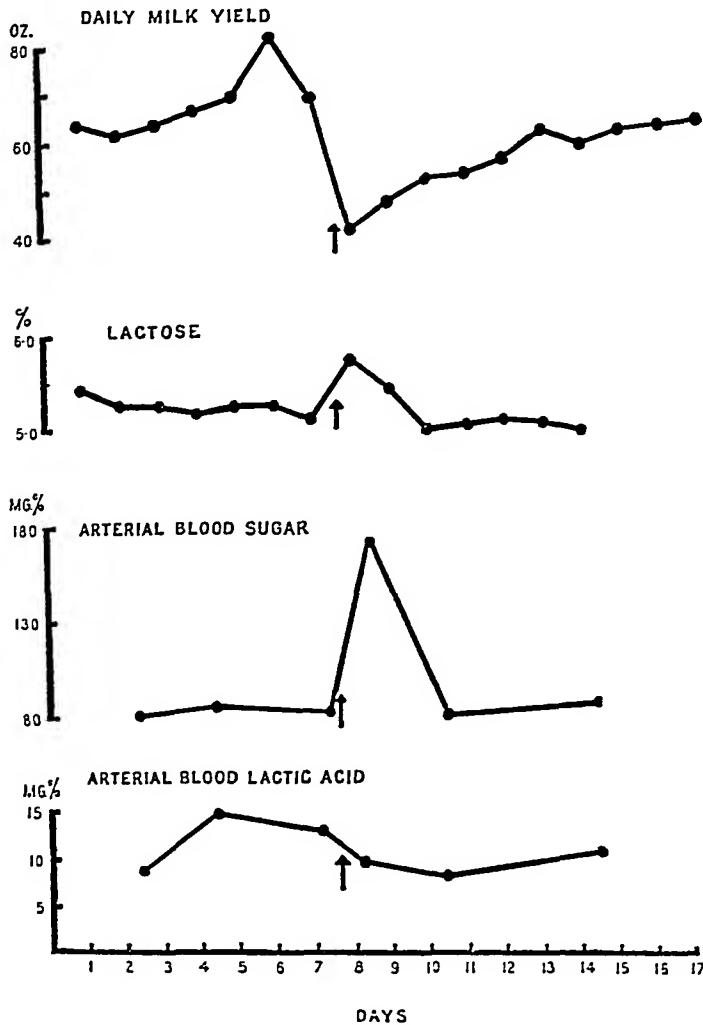
FIG. 2. Goat Ar. (experiment 2). Adrenalin tablet implanted at ↑. In this figure milk yields and lactose percentages are entered at midnight, the lactose being determined on composite samples of p.m. and a.m. milk.

55 mg. tablet of adrenalin was implanted at 4.30 p.m. on 19 June 1939. Eighteen hours later the blood-sugar was markedly raised above normal but had returned to normal 48 hours later (Fig. 2). Blood lactic acid showed no rise in this experiment. It is possible, however, that the blood lactic acid had risen and returned to normal before

the second post-implantation blood sample was taken, since Cori *et al.*
[1930 *b*], in the rat, reported that the rise in blood lactic acid following
adrenalin injection had disappeared when the blood-sugar was still high.
Milk lactose content showed a temporary rise coincident with the high
blood-sugar. The milk yield dropped sharply after the implantation,
to such a degree that the total daily output of lactose was reduced,
and slowly returned to its previous level. There was a slight post-
implantation rise in non-fatty solids but no significant change in milk
fat content.

Similar results as to the temporary rise in milk lactose and non-fatty
solids and fall in milk yield were obtained in another experiment with the
same goat (experiment 3), but on this occasion blood samples could not be
obtained from the carotid loop.

Experiment 4. Experiment 4 was carried out on goat Na. (approx.
100 lb. in weight) which was $1\frac{3}{5}$ months in milk. The milk yield was rather
irregular in the period preceding the experiment, but averaged about
60 oz. daily. Observations were made morning and evening on the milk
yield and on the percentage of non-fatty solids, fat, and lactose. Over the
greater part of the experiment blood-sugar and lactic acid were also
estimated, and determinations of these quantities were begun 5 days
before the experiment was started. A tablet of adrenalin weighing 51·2 mg.
was implanted at 2.45 p.m. on 10 May 1938. As shown in Fig. 3, the
observations indicated a great increase in the blood-sugar on the day after
the implantation. The value was still raised slightly on the second day,
$45\frac{1}{4}$ hours after implantation. Blood lactic acid was also somewhat
increased. There was a temporary increase in the percentage lactose
in the milk, associated with the rise in blood-sugar and lactic acid.
Since there was no immediate change in milk yield the implantation
resulted in an increase in the total daily yield of lactose, a different
result from that obtained in experiments 2 and 3. The percentage of
milk fat was unchanged, while non-fatty solids showed a temporary
rise.

Experiment 5. It seemed of interest to determine whether or not the
results obtained with goats could be duplicated in a cow. Accordingly,
experiment 5 was carried out on cow B1, weighing about 900 lb., and
3 months in milk. The cow was at the peak of the lactation curve with
the milk yield constant at about 27 lb. daily. Milk fat, non-fatty solids,
and lactose percentages were determined on samples from each milking,
beginning 2 days before implantation of the adrenalin tablet. Blood-sugar
and lactic acid were determined in arterial and mammary venous blood
samples. At 4.30 p.m. on 2 February 1938 tablets of adrenalin amounting
to 496 mg. were implanted. There was a rise in arterial and mammary
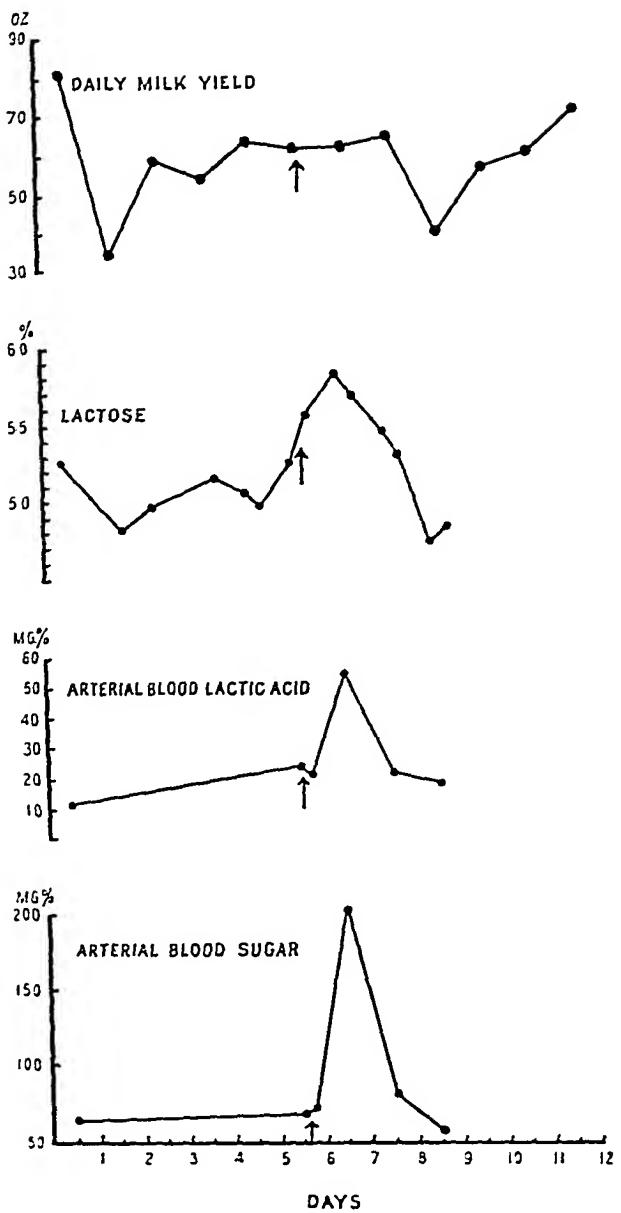
Fig. 3. Goat Na. (experiment 4). Adrenalin tablet implanted
at ↑. Results of lactose determinations are entered at the time
at which the samples were taken.

venous blood-sugar and lactic acid on the day after implantation (Fig. 4). The percentage milk lactose also showed a rise on the day after implantation (Fig. 5). There was an immediate fall in milk yield. The daily fat percentage rose, but there was no change in the daily percentage of non-fatty solids. There was a fall in both the daily yield of non-fatty solids, and also in that of lactose, due to the fall in milk yield. In other words, the decrease in the milk yield more than outweighed the rise in the percentage lactose. Further, the rise in fat percentage was not sufficient to cause a rise in total daily fat production.

It was hoped to obtain information about the uptake of glucose and lactic acid by the mammary gland from comparison of the analyses of the arterial and mammary-venous blood samples. Unfortunately, it was not found possible[1] to take more than three arterial samples by the technique used, and of these three the last was obtained too long after the venous sample to permit satisfactory comparison [see Graham *et al*, 1936]. On the day after implantation the uptake of glucose was apparently almost unchanged, while that of lactic acid was trebled, but these findings require confirmation. The figures for venous blood, however, should be reliable.

Experiment 6 (Control). Control experiments with both goats were considered advisable in order to estimate the effect of the implantation technique on blood and milk composition. Therefore, after a suitable control period in which observations were made as before, a 44-mg. tablet of paraffin wax was subcutaneously implanted at 12.30 p.m. on 26 March 1939 into goat Ar. At this time she had been 7½ months in milk. The values for blood-sugar and lactic acid, milk yield and lactose content are given in Fig. 6. It is seen that in this experiment the implantation of an inert tablet was followed by a drop in milk yield of about the same magnitude as in two of the three adrenalin experiments with this goat, indicating that in this animal, which is rather excitable, the drop in milk yield following adrenalin was due to the disturbance associated with the implantation rather than to a specific effect of the hormone.

Experiment 7 (Control). The results of an experiment with goat Na. (5 weeks in milk) in which a 43-mg. wax tablet was implanted at 4.30 p.m. on 19 June 1939 are given in Fig. 7. In this experiment lactose was determined in composite milk samples as in experiment 2. This animal is more placid than goat Ar., and in this case it is clear that the implantation had no effect on milk secretion. This result is significant in view of the fact that adrenalin implantation was not followed by a drop in milk yield in

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[1] On successive occasions it became increasingly difficult to feel the internal iliac artery through the rectal wall, probably because of the formation of haematomata after previous punctures.
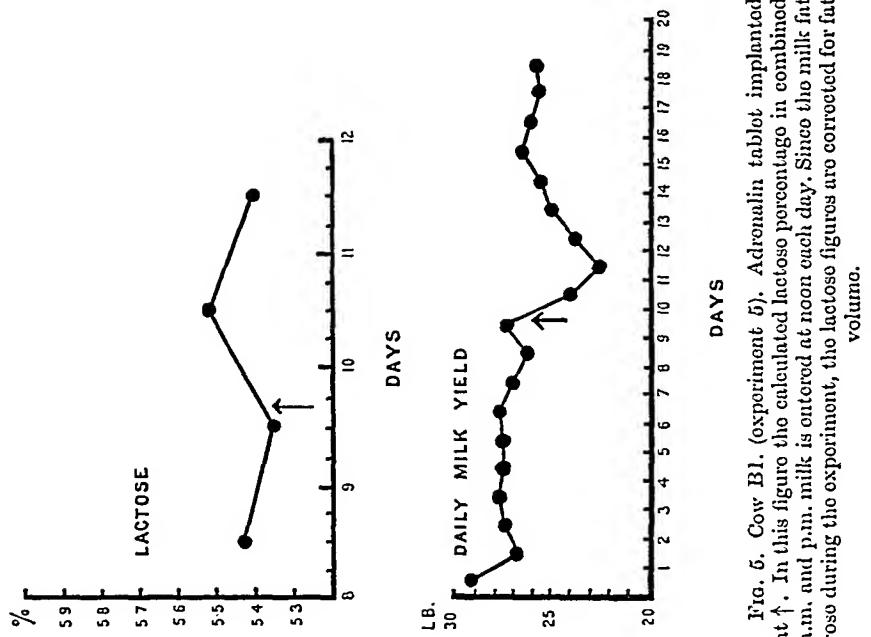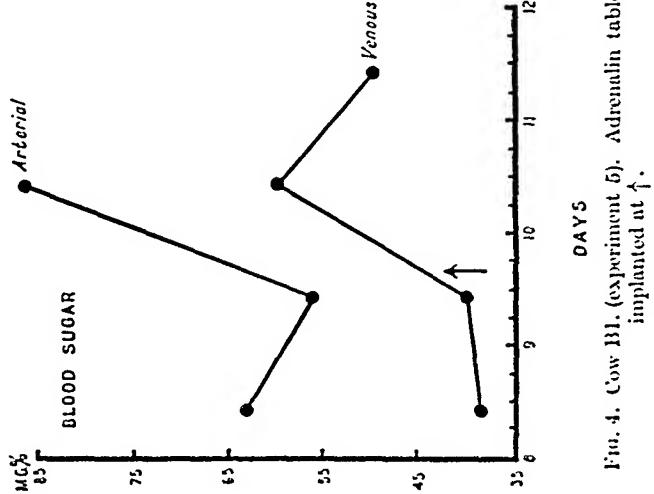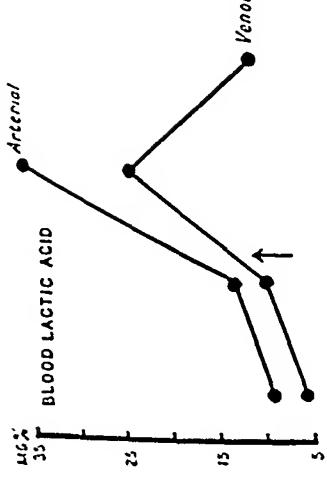
FIG. 5. Cow B1. (experiment 5). Adrenalin tablet implanted at ↑. In this figure the calculated lactose percentage in combined a.m. and p.m. milk is entered at noon each day. Since the milk fat rose during the experiment, the lactose figures are corrected for fat volume.

FIG. 4. Cow B1. (experiment 5). Adrenalin tablet implanted at ↑.

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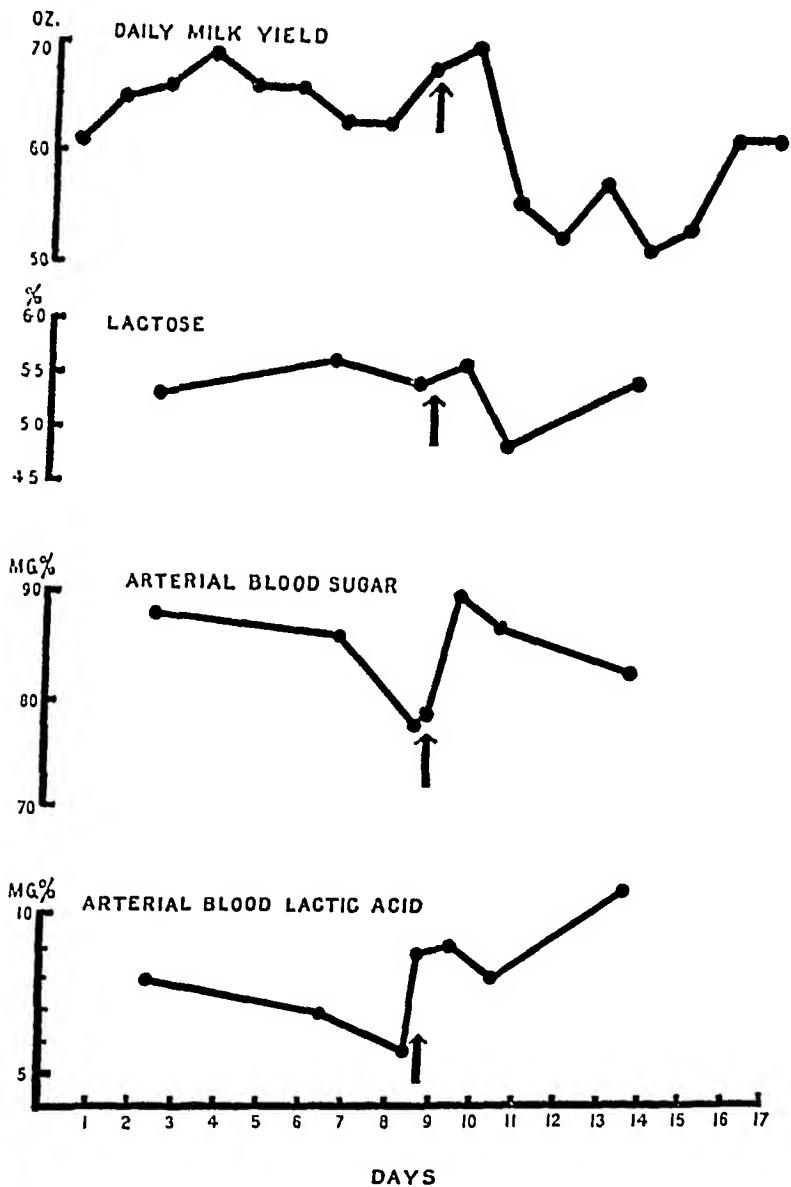
FIG. 6. Goat Ar. (experiment 6, control). Paraffin-wax tablet implanted at ↑. Results of lactose determinations are entered at the time at which the samples were taken.

this goat and supports the view that adrenalin itself does not decrease milk yield.

DISCUSSION

These experiments in the first place indicate that the tablet implantation technique of Deanesly and Parkes [1937] is useful in the case of adrenalin. In all cases the results showed that implantation of solid adrenalin was followed by hyperglycaemia of long duration which, in the most favour-

able cases (experiments 1 and 4), amounted to at least 42 hours. Unfortunately, close estimates of the duration of the hyperglycaemia can in no case be made since it was not considered advisable to puncture the exteriorized carotid arteries more frequently than once a day. None of the animals
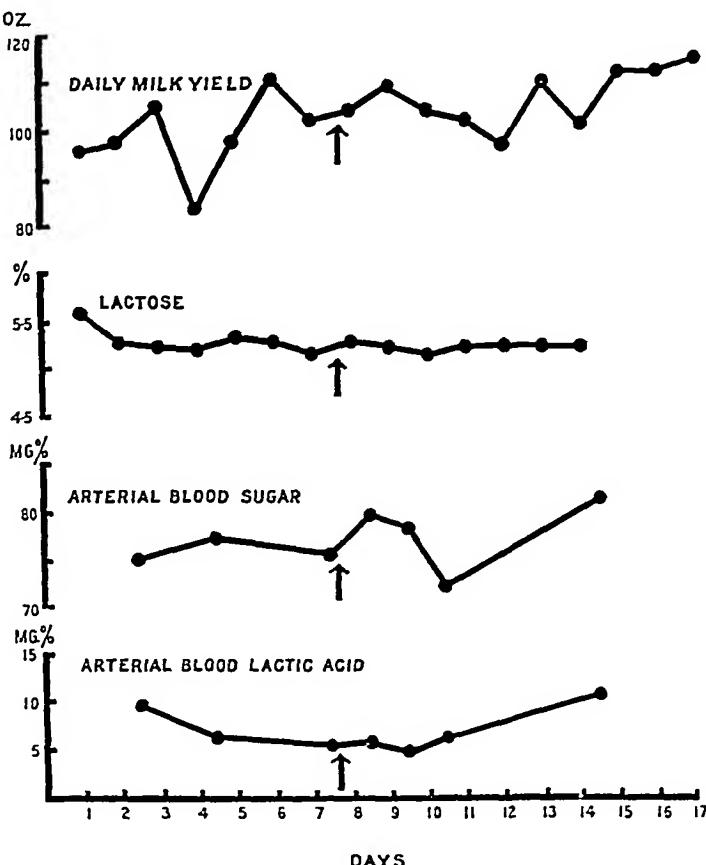
FIG. 7. Goat Na. (experiment 7, control). Paraffin-wax tablet implanted at ↑. In this figure milk yields and lactose percentages are entered at midnight, the lactose being determined on composite samples of p.m. and a.m. milk.

died from 'adrenalin shock' [see Wyman and tum Suden, 1939], though in a preliminary experiment to test this point, implantation of two 25-mg. tablets at intervals of an hour resulted in the death of the goat (a virgin female) 6 days later.

Graham, Jones, and Kay [1936] found that, in normal cows, the fall in sugar content of blood on passing through the mammary gland was positively correlated with the sugar-level of the arterial blood and also,

though this is less certain, with the milk yield. The work of Gowen and Tobey [1931] and others on the effects of inanition, and administration of insulin or phloridzin indicates that a lowering of the blood-sugar level, however produced, leads to a fall in milk yield and a diminished lactose content. It is, therefore, attractive to speculate that blood-sugar is a limiting factor in milk secretion and that if the blood-sugar level could be raised for a sufficiently long period a rise in milk yield would result. At the same time an increased lactose content might be expected.

In our experiments, in which the blood-sugar level was raised for many hours, the second expectation was realized but not the first. In every case the lactose percentage rose, but the milk yield did not increase: in three experiments, in fact, it fell sufficiently to outweigh the increased lactose content, so that the total daily lactose output was reduced. The control experiments indicate that this was not due to some other physiological effect of adrenalin but rather to trauma associated with the implantation. There were no uniform changes in milk fat-content which could be ascribed to the adrenalin implantation, but in most experiments a temporary rise in non-fatty solids content occurred, which calculation showed was due to the increase in lactose content.

In two of the three experiments in which blood lactic acid was estimated it was found to increase coincidently with blood-sugar and milk-lactose percentages. The absolute lactose production also increased in experiment 4. These findings might be interpreted as giving indirect support to Graham's [1937] view that both glucose and lactic acid are utilized by the mammary gland for the synthesis of lactose, though, on the other hand, it might be argued that they are equally consistent with the classical theory of the origin of lactose from the blood-sugar alone. In experiment 5 it would appear that, although the absolute output of lactose fell, the gland took up much more lactic acid after the adrenalin implantation, while the glucose uptake was unchanged. This anomalous result is open to suspicion, since the last arterial blood sample was not obtained within the requisite time. The venous samples, however, were obtained quickly, and these showed an appreciable rise in glucose and lactic acid, thus confirming the results of experiment 4.

In passing, it may be noted that the normal values for the arterial blood lactic acid of the lactating goat determined during the control periods in these experiments are very much lower than values reported by Houchin, Graham, Peterson, and Turner [1939], which for 28 samples ranged from 21·29 to 134·30 mg. % with a mean of 53·88 mg. %. Our 13 determinations ranged from 5·4 to 15·0 mg. % and averaged 8·56 mg. %, results of the same order as for human venous blood [e.g. Long, 1924].

It is of some interest to find that prolonged hyperglycaemia, under the

conditions of these experiments, may, in favourable circumstances, i.e. when the trauma of implantation does not lower the milk yield, lead to an increase in lactose secretion but not to an increase in the volume of milk secreted. Blood-sugar and possibly lactic acid therefore appear to be limiting factors in lactation as far as lactose synthesis is concerned, but as regards the volume of fluid secreted in unit time by the mammary gland the position is probably complicated by alterations in the blood-supply due to the action of the adrenalin.

SUMMARY

1. By the subcutaneous implantation of adrenalin tablets into goats and a cow, hyperglycaemia was maintained for many (in two cases for at least 42) hours.

2. Blood lactic acid rose appreciably during the same period.

3. Coincidently, the percentage of lactose in the milk was raised.

4. In no case was the milk yield increased, while in three experiments it fell, evidently because of trauma associated with the implantation.

5. No uniform effects on milk fat were found, but there was a tendency for the non-fatty solids content temporarily to increase.

6. It is incidentally recorded that the arterial blood lactic acid in the normal lactating goat ranged from 5·4 to 15·0 mg. %.

We are much indebted to Prof. H. D. Kay and Dr. A. S. Parkes for their interest in this work and to the latter for making and supplying the adrenalin tablets. We also desire to thank Dr. G. W. Scott Blair for determinations of milk fat and non-fatty solids.

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THE UTERUS MASCULINUS OF THE RABBIT AND ITS REACTIONS TO ANDROGENS AND OESTRO GENS

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THE uterus masculinus of the adult rabbit is a large bilobed sac lying behind the bladder; it opens into the prostatic portion of the urethra and is lined by a complex epithelium [Plate I, Fig. 1]. The degree of complexity varies in different breeds of rabbits, and of those studied the Himalayan shows the most highly developed epithelium. In this breed, complexity of the epithelium is comparable to that seen in the progestational rabbit uterus, though it is clearly different in nature and development. Hütt [1927], who discusses the earlier literature, gives an excellent description of the uterus masculinus. Some doubt exists as to its embryological origin in the rabbit. Kölliker [1879] and other nineteenth-century embryologists found that the Müllerian ducts tended to atrophy in the foetus, although traces of them might occasionally persist. Hütt concludes that it is on the whole unlikely that the uterus masculinus is of Müllerian origin. Mihalcovics [1885] considered that it develops mainly from the Wolffian ducts.

Schaap [1899] first observed the effects of castration on the uterus masculinus in two rabbits; he states that castration causes a decrease in the size of the organ. Hütt [1927], in a further investigation of castration changes, showed that in addition to shrinkage there was involution of the epithelium to a single layer of dense-staining cells. X-irradiation of a rabbit, causing destruction of the seminiferous tubules, did not produce castration changes in the uterus masculinus, so that apparently its maintenance depends on the interstitial cells of the testis. Courrier [1934] and Seeman [1937], following earlier workers, have shown that crude testicular extracts, especially from pigs, have an oestrogenic as well as an androgenic action; experiments were therefore started to determine the effects of different sex hormones on the uterus masculinus of immature and castrate rabbits, and to ascertain under what conditions full growth of the organ could be produced in the castrate.

Apart from the question whether or not the uterus masculinus of the rabbit should be regarded as an heterosexual rudiment, it seemed interesting to compare its reaction to hormones with that of the uterus masculinus in other species. Zuckerman [1936] and Zuckerman and Parkes

[1936 *a, b*] discuss the effects of oestrogens and androgens on the male reproductive tracts of different primates. Courrier and Cohen-Solal [1936] and Laqueur [1936] refer to the changes in the uterus masculinus of the guinea-pig after castration and to the cornification of the epithelium caused by oestrogens. Laqueur notes that the reaction of the uterus masculinus is slighter in uncastrated guinea-pigs; this agrees with the experimental evidence (Zuckerman and Parkes, 1936 *a*) that androgens tend to inhibit the reaction of the uterus masculinus to oestrogens.

Methods

The male rabbits used were mostly Himalayans, but included some of other breeds. Sex hormones and testicular extracts were given either as subcutaneous injections in oil or, in the case of pure substances, as compressed tablets implanted under the skin, with aseptic precautions, under ether anaesthesia. Deanesly and Parkes [1937 *b*, 1938] have shown that this may be an effective method for the administration of steroid sex hormones. It was originally thought, however [Klein and Parkes, 1937], that less wastage of sex hormones administered by injection occurred in the rabbit than in the rat, and in all the earlier experiments recorded in this paper the hormones were administered as subcutaneous oil injections. Tablets of hormone were weighed to the nearest mg. or tenth of a mg. before implantation, and dissected out, dried, and reweighed at the end of the experiment, so that the amount absorbed by the rabbit could be determined.

Epithelial Development of the Uterus Masculinus in the Normal and in the Cryptorchid Rabbit

The uterus masculinus was examined histologically in 9 normal rabbits, 3 Dutch, 2 Lops, and 4 Himalayans; of these 2 Dutch and 2 Himalayans were immature. In Himalayans weighing 1,100 g. and 1,340 g. the uterus masculinus is fairly large, though it has not quite reached its adult size. The epithelium, however, shows little or no glandular development, and over a great part of the surface it is only single-layered, as after castration. The uterus masculinus of the smallest immature Dutch rabbit (900 g.) had an epithelium single-layered in parts, but elsewhere having projecting papillae of irregular columnar cells, though no glandular invaginations had been formed; at 1,100 g. another Dutch rabbit had a glandular epithelium resembling that of the adult, although not fully developed.

It seems clear, therefore, that the uterus masculinus only gradually reaches its full development in the normal rabbit and that it grows in size before the epithelium starts active proliferation. Hütt [1927] gives the dimensions of the organ in the adult as 3·5 cm. in length and 1·5 cm. in

width. No measurements were made during the present work as it w:
observed that the organ underwent shrinkage during dissection and c
fixation.

The condition of the uterus masculinus was examined in two cryptorchi
Himalayan rabbits. From each of these animals one immature testis wa
removed when the body-weight was 800 g., while the other testis was witl
drawn into the abdomen and attached to the abdominal wall. Thes
rabbits were killed 160 and 203 days after operation and weighed 1,520 g
and 1,780 g. In neither case was the uterus masculinus of normal size, nc
was the epithelium fully developed, although the prostate was conspicuou
and showed no castration effects. In view of Hütt's finding that the condi
tion of the uterus masculinus appeared to depend on the interstitial cell
of the testis, this was an unexpected result. A possible explanation is tha
more secretion is required to maintain the uterus masculinus than to main
tain the prostate, and that the one surviving cryptorchid testis was
inadequate for the purpose.

The Effect of Oestrogens and Androgens on the Uterus Masculinus of Normal Adult and Immature Rabbits

Three old male rabbits, 1 Himalayan and 2 Lops, were each given a
single injection of 2 mg. oestrone benzoate in oil, a preparation which,
owing to its slow absorption, exerts a gradual effect on the organism for
a considerable period. These rabbits were killed respectively 13, 20, and
33 days after injection; the uterus masculinus appeared normal and showed
neither breakdown of the epithelium nor metaplasia of the glands.

A further experiment was carried out in which two tablets of oestrone
weighing 86 mg. and 90 mg. were implanted subcutaneously in an adult
Himalayan male rabbit, which was killed 40 days later. Since prolonged
treatment with oestrogens in tablet form inhibits the gonadotrophic secre-
tion of the pituitary gland in rats [Deanesly, 1939], it was thought that
degeneration of the testes would occur and that this would affect the
accessory sexual organs. If the uterus masculinus depended primarily for
maintenance on an oestrogenic rather than on an androgenic secretion,
then it might remain normal and not revert to the castrate level. Actually
it was found at autopsy, as expected, that after the absorption of 5 mg.
oestrone the testes were shrunken and flabby, while the uterus masculinus
was large; its epithelium, however, was of a type not before seen, less com-
plex than the normal, though far better developed than in the castrate.
Since it contained abundant leucocytes, it probably represented a stage
of regression from the full, normal proliferation.

Other experiments were carried out on immature rabbits with mainly
negative results. Five Dutch rabbits averaging 815 g. received 15 μg.

oestrone in 7·5 c.c. nut oil over 5 days; the uterus masculinus showed no appreciable reaction. A larger Dutch rabbit, 1,400 g., received 60 μg. oestrone over 5 days, and here too the uterus masculinus, already fairly well developed in the control animal, seemed unaffected.

Five tablets of oestrone totalling 66 mg. were implanted subcutaneously in an immature Himalayan rabbit, which was killed 28 days later. The uterus masculinus at death was large, but the epithelial proliferation was very slight and did not approach the adult condition. These results indicated that oestrone could enlarge or maintain the size of the uterus masculinus, but could not stimulate the production of an adult epithelium in a normal immature animal.

In later experiments 2 immature male rabbits, 1 Himalayan and 1 Dutch, weighing about 800 g. at death, received testosterone tablets of 44 mg. and 29 mg. respectively for 30 and 37 days. About one-third of the tablet was absorbed in each case, but neither rabbit showed anything approaching an adult uterus masculinus, although each prostate had responded to the male hormone. The uterus masculinus showed some inflation, but the epithelium was comparatively undeveloped.

The Effects of Injection of Oestrogens, Androgens, and Other Substances on the Uterus Masculinus of the Castrated Rabbit

Effects of injections. Simultaneously with the experiments already described, others were carried out on castrated rabbits. Most of these were castrated as immatures; Hütt's observation that the epithelium of the uterus masculinus remained undeveloped was confirmed and it was noted that urethral epithelium tended to invade even the anterior regions of the organ. Plate I, Fig. 2 shows part of the uterus masculinus of a Himalayan rabbit castrated when immature and killed 6 months later. Immature castrate rabbits were injected with oestrone at varying periods after castration. Two Himalayans received 100 μg. and 500 μg. oestrone respectively, given in 5 oil injections over 10 days, and another Himalayan received 600 μg. in 30 daily injections. These animals were compared with a castrated control belonging to the same group, and it was found that oestrogens had caused the shrunken uterus masculinus of the castrate to grow to its normal size, but had failed to stimulate the production of a normal epithelium. There was enlargement of the muscular wall of the uterus masculinus and congestion and oedema in the sub-epithelial tissues, but the epithelium showed only slight growth of the type illustrated in Plate I, Fig. 3, not unlike that found in the normal immature rabbit. Larger doses of oestrone, 0·1 mg. daily for 11 days and 0·5 mg. daily for 10 days (Plate I, Fig. 3), gave similar results in two other castrate Himalayans.

A second group of castrate rabbits received injections of testosterone

or testosterone propionate in oil; 6 mg. and 10 mg. of testosterone propionate in oil were given over 10-day periods to Himalayans, and 7·5 mg. of testosterone over 30 days to a Lop rabbit. In none of these animals did the uterus masculinus show any enlargement or alteration from the castrate type. Experiments on two other castrated rabbits showed that combinations of oestrone and testosterone, given either simultaneously or successively, produced results similar to oestrone injections alone. It was thought, after these experiments, that the epithelium of the uterus masculinus might depend on some hitherto undiscovered oestrogenic substance in the testis, and an attempt was made to restore castration changes in a series of 8 Himalayan rabbits by different fractions of pig testis extract PT4C, injected in oil for periods varying from 14 to 30 days. This is an extract with comparatively strong oestrogenic, but weak androgenic action [Emmens and Parkes, 1938]. Most of the fractions enlarged the uterus masculinus, and the crudest of them stimulated the production of a thickened epithelium, perhaps indicating the beginnings of glandular development, but in no case was anything approaching a normal adult epithelium formed.

A further test was carried out with *trans*-androstenediol, an artificially produced substance known to have both oestrogenic and androgenic properties [Butenandt, 1936; Deanesly and Parkes, 1937 a; Emmens, 1938]; the castrate rabbit was injected daily and received 27·5 mg. over 11 days, but the uterus masculinus showed only a very slight oestrogenic response. Another rabbit received a combination of 150 μg. of oestrone given over 6 days followed by 2 mg. of progesterone over 5 days, but the latter substance appeared to have no effect on the uterus masculinus.

The most striking feature of these results was the failure of testosterone or testosterone propionate to affect the uterus masculinus, although previous experiments had shown that a dose of 20 mg. of testosterone would cause growth of the immature rabbit uterus [Deanesly and Parkes, 1937 a], in spite of its weak oestrogenic action in the rat. Since it was known that methyl testosterone showed a progesterone-like activity in the female rabbit [Klein and Parkes, 1937], the effect of this substance on the uterus masculinus was next examined.

Effects of tablet administration. Methyl testosterone was implanted subcutaneously in the form of compressed tablets (Table I), and it was found that in castrated rabbits they caused striking proliferation and growth of the uterus masculinus. Testosterone also produced marked effects by the same method of administration, and, since the injection of 10 mg. of testosterone propionate produced no reaction in the uterus masculinus, it must be assumed that injected androgens are liable to wastage in the rabbit and that the dosage was too low. Three experiments were

carried out with tablets of methyl testosterone in which the amounts absorbed were 25, 27, and 18 mg. during 40, 20, and 15 days respectively (Table I). Examination of sections through the uterus masculinus from

Table I. *Implantation of male hormone tablets into castrate rabbits*

No.	Substance	Amount implanted mg.	Days	Total absorbed mg.
QOM 50	Methyl testosterone	101	40	25
51	,, ,,	99+76	20	27
70	,, ,,	83+65	15	18
82	Testosterone	84	75	40
81	,,	105+68	40	70
55	,,	144+98	30	37
71	,,	123+82	23	32

the corresponding castrate rabbits showed that after 40 days the proliferation hardly differed from that of a normal adult (Plate I, Figs. 1 and 4), while after 20 days there was a well-developed epithelium differing from the normal in its less complex glandular invaginations and in the cytoplasmic borders still persisting in the gland cells. Presumably this represents an intermediate stage of epithelial development. In the last rabbit of this group, which absorbed 18 mg. of methyl testosterone over 15 days, epithelial development was much less advanced, although glandular invagination had begun. Since the androgenic substance in these experiments must stimulate the growth of the fibromuscular wall of the uterus masculinus as well as that of the epithelium, it is comprehensible that the process should be a slow one requiring a fairly high amount of hormone. In the normal rabbit it is probable that the oestrogenic hormone of the testis contributes to the development of this organ which, as already mentioned, enlarges before full epithelial proliferation occurs.

In the experiments with testosterone the largest amount absorbed (70 mg. in 40 days) restored the uterus masculinus to its normal size and stimulated a glandular epithelium, with perhaps less complex invaginations than the normal adult uterus masculinus, but of an essentially similar type. After 75 days tablet implantation, when the daily amount of testosterone absorbed was only a third as much as in the previous rabbit, the epithelium of the uterus masculinus differed more from that of the normal adult. In the two other castrated rabbits receiving smaller doses of testosterone over shorter periods, the epithelium had only begun to show glandular proliferation.

These experiments indicate that the epithelium of the uterus masculinus responds to androgens but has a relatively high threshold requirement. In the normal animal it is probably stimulated not only by testosterone, but by other substances possessing androgenic or oestrogenic activity or

both, secreted by the testis. If testosterone is given alone, large doses a
required to restore the organ in the castrate. In a single experime
tablets of both oestrone and testosterone were implanted into the san
castrate rabbit for 40 days; the uterus masculinus was very large at deat
but the epithelium, though glandular, was much simpler than in the norm
adult. Probably in this rabbit the amount of testosterone absorbe
(18·5 mg.) was inadequate for complete restoration of the epithelium.

SUMMARY

Hütt [1927] showed that, after castration, the large uterus masculinu
of the adult rabbit underwent shrinkage and its complex epithelium re
gressed to a single, mainly single-layered type. In the present experiment
oestrogenic and androgenic substances in various combinations and crud
testicular extracts have been administered to rabbits in attempts to stimu
late growth and development of the uterus masculinus in castrated o
immature males. It was found that small or large quantities of oestrogen
caused conspicuous growth of the organ, but the epithelium could only b
restored to normal by the administration of methyl testosterone or testo
sterone in relatively large amounts (25 mg. or 70 mg. in 40 days), by th
subcutaneous implantation of tablets.

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FIG. 1. Normal adult male.

FIG. 2. Immature castrate male in which three types of epithelium can be seen: on the left urethral epithelium, then epithelium with small papillae, and then single-layered dark-staining epithelium.

3. Immature castrate male after the injection of 0.5 mg. oestrone in oil, daily for 10 days, showing oedema and congestion of the stroma, but hardly any epithelial growth.

FIG. 4. Immature castrate male after absorbing 25 mg. of methyl testosterone in 10 days.

MODIFICATION OF THE EFFECTIVENESS OF GONADOTROPHIC EXTRACTS

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DURING the last few years gonadotrophic extracts from various sources have been tested in many ways, in attempts to determine the number of active principles involved and the maximum activity of given preparations. It has been found that the effect of two extracts, when given together, may be much greater than the sum of the effects when they are administered singly. This is ascribed by many writers to an improved balance in the combined extract, of two different gonadotrophic principles, one follicle-stimulating and the other luteinizing. Other writers, however, especially Saunders and Cole [1938], regard the question of different hypophyseal principles as still unsettled, and attribute the greater effectiveness of combined extracts to delayed absorption of the active principle, since it is known that the addition of metallic salts and other inert substances may greatly increase the effectiveness of hypophyseal gonadotrophic preparations. In the present paper it is proposed to review some of the work on this subject, and to record tests carried out over several years on modification of gonadotrophic effectiveness.

Method

The technique was the same as that previously described [Deanesly, 1935]; immature female rats were used, weighing 40–50 g. at the beginning of the experiment, with ovaries averaging 10 mg. Unless otherwise stated, each test was made on 10 rats, which were given single subcutaneous aqueous injections daily for 5 days and were killed on the 6th day. The ovaries were dissected out, fixed in Bouin's fluid, and each pair weighed on a torsion balance after transference to 70% alcohol. The weight recorded for the ovaries in the tables is the average for the group. The dose of gonadotrophic extract is expressed as the total dose per rat; the daily dose injected in 1 c.c. is, therefore, one-fifth of the total.

The experiments of other workers will be referred to in the individual sections of the paper under the appropriate headings.

Effect of Division of Dose and Addition of Metallic Salts and Inert Substances to Gonadotrophic Extracts

Previous work on hypophyseal gonadotrophic extracts. Since the action of certain gonadotrophic extracts, like that of oestrogenic and androgenic substances, can be modified by alterations in the method of administration, experiments on divided dosage are classed with experiments involving the addition of inert substances to extracts.

Fevold, Hisaw, Hellbaum, and Hertz [1933 a] first referred to the augmenting action of an inert substance on a pituitary gland extract; they stated that the precipitation of their follicle-stimulating extract in tannic acid and the injection of the tannate increases the ovarian response owing to more gradual absorption.

Maxwell [1934] then showed that if zinc sulphate solution was combined with a gonadotrophic extract before injection, there would be a marked increase in the ovarian response in immature female rats. He added zinc sulphate in a daily dosage of 3 mg. per rat to extracts of sheep and ox hypophyses and obtained a definite augmentation in the weights of ovaries, correlated with vaginal opening and corpora lutea formation, usually absent in rats treated with these somewhat inactive preparations. Maxwell concluded that his results were entirely due to delay of absorption of the active principle since (1) zinc sulphate injected separately had no gonadotrophic action, and (2) removal of the zinc left the gonadotrophic preparation with approximately its original potency. Isolated experiments showed that tungstic acid or aluminium salts would also augment the action of hypophyseal extracts. Maxwell's work has been repeated and extended by Bischoff [1938], Fevold, Hisaw, and Greep [1936, 1937], de Fremery and Scheygrond [1937], de Fremery [1938], Emery [1937 a, b], Casida [1936], Hellbaum [1936], McShan and Meyer [1937], and Saunders and Cole [1936, 1938]. Fevold et al. [1936], finding that mature rabbits could be induced to ovulate by extracts of yeast, tested the effect of the latter on immature rats in combination with hypophyseal extracts, and obtained augmentation of ovarian weight. Further experiments showed that this augmentation could also be obtained from the inorganic constituents of the yeast, including copper. It was found that copper produced more augmentation than zinc under certain conditions, whereas iron was less active than zinc. Copper, however, differed from zinc in being ineffective in augmenting the follicle-stimulating fraction in hypophysectomized rats, although it would augment the follicle-stimulating fraction in such animals if combined with the luteinizing fraction. Copper salts also differed from zinc in causing ovulation in the mature oestrous rabbit. Fevold et al. [1936], therefore, suggest that the biological actions of zinc

and copper differ qualitatively. Bischoff [1938], discussing these results in the light of further experimental work, shows that the amount of augmentation produced by zinc is greatest when the solution is alkaline. With copper, on the other hand, which produces more necrosis of the tissues at the site of injection, the pH of the solution does not materially affect the degree of augmentation, which is greater at pH 6 or 8·5 than with zinc. Further tests showed that the active substance is precipitated by both zinc and copper at pH 8·5. Saunders and Cole [1936] confirm Maxwell's experiments and state that they obtain an average additional increase of about 100% in ovarian weight, by adding zinc sulphate to a crude pituitary extract. In a more recent paper [1938] the same authors study the amount of zinc required to produce maximum augmentation with a given hypophyseal extract (Evans's synergist prepared from sheep or ox hypophysis). Saunders and Cole do not state the pH at which their uncentrifuged solution was injected, but their results indicate that the maximum augmentation produced by zinc brings the weight of the ovaries in these experiments to 71 mg., or rather more than double the weight found when the hypophyseal extract is given alone.

Experiments on division of dose and addition of zinc sulphate to hypophyseal gonadotrophic extracts. In the first set of experiments a total of 15 mg. zinc sulphate per rat was added to hypophyseal gonadotrophic extracts from different species (Table I). The addition of the zinc sulphate solution to the hypophyseal extract generally caused the formation of a precipitate at pH 7·5, to which the solutions were adjusted. In most experiments this precipitate was injected with the solution, but in certain tests, indicated in the table, it was separated by centrifuging and the supernatant fluid alone was injected.

Some of the hypophyseal extracts showed comparatively low maximum activity on the immature female rat. In the case of the pig pituitary gland extract AP51, it was found that if injections were given twice instead of once daily, a total of 25 mg. per rat would produce ovaries averaging 37 mg. instead of 22 mg. (Table I). Apparently some of the activity in this preparation was wasted through too rapid absorption. This agrees with the conclusions of Maxwell [1934] and de Fremery and Scheygrond [1937] concerning the augmentation of the action of pig pituitary gland by zinc sulphate.

The results in Table I show that hypophyseal extracts from pig, sheep, and ox, but not from horse, are augmented by the addition of zinc sulphate, the average weight of the ovaries after a given dose being often more than doubled. With pig and sheep extracts the increased activity due to the zinc appears to be independent of the precipitate; with two different extracts of ox pituitary gland, however, a contrary result was

obtained; at pH 6 most of the active substance disappeared from the supernatant fluid. Test 72 (Table I) shows that if this precipitate is taken up with water and neutralized, the active substance is recovered Further tests, each on 5 rats, confirmed this observation and showed that the precipitate contained very little zinc.

In another set of experiments (Table II) an attempt was made to find the maximum augmentation of pig hypophyseal extract AP74, and the amount of zinc necessary for it. All solutions containing zinc sulphate were centrifuged and only the supernatant fluid was injected; a record was kept of the dry weight of the precipitate in tests 54–6. Tests 52–4 illustrated the usual results with this type of gonadotrophic extract— failure of the ovarian response to increase proportionately to the dose above a certain limit (tests 52, 53), and doubling of the response when zinc sulphate was added to the lower dose of AP74 (test 54). There is no further increase in ovarian weight when zinc sulphate is mixed with the higher dose of AP74 (test 57), indicating that the response to the extract is still a limited one, whether or not zinc is added.

In tests 54–6 a standard amount of the gonadotrophic extract was com- bined with varying amounts of zinc sulphate; although the dry weight of the precipitate removed by centrifuging was much the same in tests 54 and 55, the ovarian response in test 55 was markedly decreased, indicating that the better ovarian response in test 54 was due to delay in absorption caused by the residual zinc in the supernatant fluid. Shortly after these experiments had been made, Saunders and Cole [1938] recorded somewhat similar results with a hypophyseal extract described as 'pituitary synergist'. These writers, using very small groups of rats, found that the increased response due to the addition of zinc tended to diminish when the dose was decreased, especially with a total dose below 2·5 mg.

The present experiments confirm the conclusions of Maxwell [1934] and Saunders and Cole [1938]; they indicate that the increase in effectiveness due to zinc sulphate is probably the result of delayed absorption and is certainly not due to any change in the gonadotrophic extract. The results are most striking in the case of ox pituitary gland, which, administered alone, has only a very slight effect in the immature female rat. With the addition of zinc sulphate this extract will cause vaginal opening and corpora lutea formation.

The fact that the addition of zinc sulphate failed to augment the action of horse hypophyseal extract (Table I), or human hypophyseal extract [de Fremery and Scheygrond, 1937], suggested that the reaction of these substances (as with the pregnant mare serum extract referred to below) might be little affected by the conditions of absorption. If this were the case, then a given dose should produce approximately the same reaction

Table I. *Effect of 15 mg. zinc sulphate on response to hypophyseal gonadotrophic extracts*

Test no.	Extract. Source and no.	Total dose mg.	Total zinc sulphate mg.	Ovarian weight mg.	No. of rats.
R	Pig AP51	25	—	22	10
41	,,	25	15	55	10
R	Pig AP63D ·	12·5	—	28	5
R	,,	25	—	56	5
42	,,	12·5	15	49	10
43	,,	12·5	15*	63	10
46	Sheep AP45D	15	—	45	20
48	,,	15	15	98	10
47	,,	15	15*	105	10
49	Ox AP24B	15	—	15	10
50	,,	15	15	25	10
51	,,	15	15*	15	10
R	Ox AP15B	50	—	15	10
67	,,	25	15	27	10
69	,,	25	15*	9	10
72	,,	25	15†	27	9
R	Horse AP61B	2·5	—	58	10
64	,,	2·5	15	58	10

* Centrifuged: supernatant fluid only injected.
† Centrifuged: supernatant fluid removed; precipitate taken up with water and injected.

Table II. *Effect of various amounts of zinc sulphate on effectiveness of pig hypophyseal extract AP74D*

Test no.	Total dose AP74D mg.	Total dose zinc sulphate mg.	Dry weight of precipitate* mg.	Ovarian weight mg.
52	25	—	—	28
57	25	15	—	46
53	12·5	—	—	25
54	12·5	15	52	44
55	12·5	5	60	26
56	12·5	1·75	40	24

Ten rats in each group.

* The precipitates were formed when 150 mg. AP74D extract was combined with 150 mg., 60 mg., and 21 mg. of zinc sulphate (tests 54–6 respectively) in 60 c.c. aqueous solutions.

in a 5-day test, whatever the number of injections in which it was administered. Tests on two groups of 5 rats with horse hypophyseal extract AP61 (2·5 mg.) (cf. Table I), and on two further groups of 5 rats with human hypophyseal extract AP91 (0·5 mg.) indicated clearly, however, that with one injection instead of 5 the reaction was much reduced; the ovarian response to the dose of AP61 decreased to 13 mg. from 55 mg., and that to AP91 to 20 mg. from 65 mg.

Previous work on urine of pregnancy extracts. It was observed by several workers in the course of early experiments that the response to urine of pregnancy extract appeared to be unaffected by divided dosage [Evans and Simpson, 1929; Bischoff, 1936] or by the addition of zinc [Maxwell, 1934; de Fremery and Scheygrond, 1937]. On the other hand, Shelesnyak and Engle [1932] showed that the response to urine of pregnancy extract could be affected by the method of administration, and both Fluhmann [1934] and Deanesly [1935] found that in certain circumstances a dose of urine of pregnancy extract produced a greater response if spread over 10 rather than over 5 days. More recently Hamburger and Pedersen-Bjergaard [1938] have shown that, in 100-hour tests, a single subcutaneous or intravenous injection of urine of pregnancy extract will produce a much lower ovarian response than will the same dose administered in 5 subcutaneous injections during the first 48 hours. Emery [1937 *b*] reports some augmentation of the action of urine of pregnancy extract by zinc sulphate, but only if the extract is given intra-peritoneally.

Effect of divided dosage and addition of zinc sulphate on urine of pregnancy extract. Two group tests were carried out in this laboratory in which the urine of pregnancy extract UP10 was used; a dose-response curve for this extract has already been published [Deanesly, 1935]. Half a milligram will produce ovaries weighing 26 mg. in the usual 5-day test; if the same dose is given in twice daily injections, or if 15 mg. of zinc sulphate per rat is added to the solution, similar ovarian weights are obtained, thus confirming the conclusions of previous workers that the behaviour of urine of pregnancy extract in tests of this kind differs from that of hypophyseal extracts.

Previous work on pregnant mare serum extract. Cole, Guilbert, and Goss [1932] report that one dose of mare serum is as effective as 8 doses in a 4-day test. Saunders and Cole [1938] report failure to augment the action of pregnant mare serum extract with zinc sulphate or other inert substances. Hamburger and Pedersen-Bjergaard [1938] show that in 100-hour tests a single subcutaneous or even a single intravenous injection of pregnant mare serum extract is as effective as 5 subcutaneous injections spread over the first 48 hours. It is clear, therefore, that in this test pregnant mare serum extract gives a much better response to one injection than urine of pregnancy extract.

Effect of divided dosage and addition of zinc sulphate on pregnant mare serum extract. In a 5-day test on 2 groups of 5 rats a dose of 1·5 mg. pregnant mare serum extract MS1 produced ovaries averaging 77 mg. when given as one injection, and 62 mg. when given as usual in 5 injections. This confirmed the findings of earlier workers that such extracts showed no loss of activity when administered as a single injection. The other tests carried

out in this laboratory were on two different preparations, PMS16 and PMS18; a note on their activity has been published by Rowlands [1938]. Table III shows that, in 3 tests with different dosage, the solution containing zinc sulphate produced smaller ovaries than were found in the corresponding controls.

Table III. *Effect of zinc sulphate on response to pregnant mare serum extract*

Test no.	Extract no.	Total dose mg.	Total zinc sulphate mg.	Ovarian weight mg.
63	PMS16	0·5	—	31
62	„	0·5	15	18
30	PMS18	1·0	—	103
68	„	1·0	15	80
35	„	0·25	—	31
74	„	0·25	15	19

Ten rats in each group.

Discussion. In a consideration of what may influence the effectiveness of gonadotrophic extracts, at least three factors must be clearly differentiated: (*a*) the extent to which the animal's own pituitary becomes involved in tests on intact animals; (*b*) the rate of absorption from the site of injection; and (*c*) the rate of destruction or excretion after absorption. The importance of these factors and their susceptibility to alteration will vary in different tests. Extracts depending largely for their gonadotrophic effectiveness on the co-operation of the animal's own pituitary gland will not be much influenced by agencies which only modify their direct effect on the gonad, and the converse will hold for extracts whose action is in the main a direct one on the gonad. Extracts whose activity is preserved by a low rate of excretion or destruction will clearly not acquire increased effectiveness through a slowing of the rate of absorption from the site of injection. These principles may be applied to the experimental results recorded above.

The augmentation of the action of hypophyseal extracts from ox, sheep, and pig by the addition of zinc sulphate and inert substances appears to be satisfactorily explained as the result of the more gradual absorption of the active gonadotrophic substance, as originally suggested by Maxwell [1934]. It remains uncertain why the action of human hypophyseal extract [de Fremery and Scheygrond, 1937] and horse hypophyseal extract (Table 1) is not augmented in the same way. These two extracts resemble each other in being of a more 'follicle-stimulating' type than those known to be augmented by zinc, and for them the usual test may ensure absorption at something approaching the optimum rate.

higher than the sum of the increases in weight produced by the extracts acting alone. Only in one of these tests were the solutions mixed before injection. These results indicate a possible synergism between horse pituitary gland and urine of pregnancy extract.

Table IV. *Effect of combining urine of pregnancy extract UP10 with horse pituitary extract AP41D*

Test no.	UP10 total dose mg.	AP41D total dose mg.	Ovarian weight mg.	No. of rats
D	0·5	—	26	20
106	—	0·5	28	10
101	—	1·25	70	10
100	0·5	1·25	108	9
107	0·5	0·5	62	10
112*	0·5	0·5	69	9

* Solutions mixed before injection.

Synergism between sheep pituitary gland and urine of pregnancy extract has been so frequently described in the literature, that a more extensive series of tests was next carried out with different and more active preparations than those used in the earlier work (Table V). The urine of pregnancy extract UP27 produced 40 mg. ovaries with a total dose of 0·1 mg.; 10 times this dose caused no further significant increase in ovarian size. The sheep hypophyseal extract AP66D produced 40 mg. ovaries with a total dose of 10 mg., and the injection of twice this amount caused no further ovarian enlargement. Sheep hypophyseal extracts nor-

Table V. *Combination of various amounts of urine of pregnancy extract UP27 with sheep hypophyseal extract AP66D*

Test no.	UP27 total dose mg.	AP66D total dose mg.	Ovarian weight mg.
R	0·05	—	28
R	0·10	—	40
78	1·00	—	41
79	—	10	43
80	—	20	40
73	0·05	10	42
71	0·10	10	48
70	0·20	10	65
66	0·50	10	83
65	1·00	10	84

Ten rats in each group.

mally have a very limited action on ovarian weight, and this particular extract was the most active of ten different ones tested in this laboratory. It was thought that synergism between the two extracts might be more readily demonstrated if they were mixed before injection, and if an amount

of urine of pregnancy extract was used in excess of the amount which would give the maximum ovarian response. The results in Table V show, however, that although the ovarian weights increase up to a point with excess dosage of UP27, yet they never exceeded, and in 3 tests out of 5 fell below, the sum of the weights produced by the two extracts independently.

It would, therefore, appear that sheep hypophyseal extracts (other than those partially fractionated into follicle-stimulating and luteinizing fractions) do not show synergism in combination with urine of pregnancy extract. This conclusion appears to agree with that of Fevold [1939], the present theory being that pregnancy urine extract as a strong 'luteinizing' substance is not complemented in its action on the ovary, except by a predominantly 'follicle-stimulating' fraction or extract. Of the latter type is the horse hypophyseal extract, which showed indications of synergism with pregnancy urine extract in the earlier experiments (Table IV).

Combinations of different gonadotrophic extracts with extract of urine of menopause. Since extracts of menopause urine show slight gonadotrophic activity, which is wholly or mainly 'follicle-stimulating', they might be expected to show synergism in combination with urine of pregnancy and other 'luteinizing' extracts, as reported by Leonard and Smith [1934] and Evans and Simpson [1935]; but most of the present experiments made with this substance gave negative results. The extract used, Path. U31G, injected alone into immature rats in a total dose of 240 mg., led to very slight enlargement of the ovaries, which weighed 11 mg.; they showed some evidence of histological stimulation, corpora lutea being present in 2 rats out of 10. If the total dose was halved, the histological reaction was purely follicular. This dose (120 mg.), given in conjunction with urine of pregnancy extract (0·5 mg. UP10), caused no augmentation of the normal response. On the other hand, in conjunction with two pig hypophyseal extracts AP51 and AP53D, the menopause urine extract produced a marked augmentation of ovarian weight. These extracts, when given alone in doses of 25 mg., will produce ovaries averaging 22 mg. and 23 mg., but averaging 50 mg. and 51 mg. when given in conjunction with 120 mg. of menopause urine extract, the two solutions being mixed before injection. The significance of this is uncertain, since the action of pig hypophyseal extract can equally be augmented by divided dosage or by zinc sulphate. On the other hand, sheep hypophyseal extract AP45D, whose activity is augmented by zinc (Table I), showed only doubtful augmentation with menopause urine extract, so that the two reactions may be quite different in nature. The other sheep hypophyseal extract showed no augmentation in combination with Path. U31G.

Inhibition of the Action of One Gonadotrophic Extract by Injection in Combination with Another

Previous work. In a number of the earlier experiments with gonado-
trophic extracts it was observed that the reactions produced in the hypo-
physectomized rat [Smith, 1927], or immature female rat [Evans an
Simpson, 1928], by pituitary implants, were partially or wholly inhibite
if extract of ox hypophysis was given simultaneously, usually by the intra-
peritoneal route. Reiss, Selye, and Balint [1931] showed that the effec
of pregnancy urine extract could be similarly inhibited in rats. Leonar
[1934 a] later reported inhibition of the ovarian weight increase caused b
pregnancy urine extract, given subcutaneously, if ox or sheep pituitar
gland was given intra-peritoneally at the same time. Leonard, Hisaw
and Fevold [1935], using the same type of test, found that out of severa
extracts the luteinizing fraction of sheep pituitary gland, given intra-
peritoneally, was the most effective inhibitor of the weight increase cause
by subcutaneous injection of pregnancy urine extract. The follicle
stimulating fraction of sheep pituitary gland, however, also caused som
inhibition, while follicle-stimulating urine extract proved to be synergistic
and increased the weight of the ovaries in combination with urine o
pregnancy extract. Collip and Williamson [1936] reported that intra
peritoneal injection of ox, sheep, and pig hypophyseal extracts usuall
gives a negative result; if, however, intra-peritoneal injection of ox hypo-
physeal extract is combined with subcutaneous injection of the same
extract, the usual effect of the latter in increasing the weights of the
ovaries is completely inhibited. Freud [1937 a, b; 1938] reports results
similar to those of Collip and Williamson [1936]; ox anterior pituitary gland
extract has no action when given intra-peritoneally by itself, and will in-
hibit the action of pregnancy urine extract when the latter is given sub-
cutaneously with it. Evans, Korpi, Pencharz, and Simpson [1936] carried
out further experiments on these lines, and discuss the purification of the
inhibiting substance, which they call the gonadotrophic antagonist. They
describe its preparation from ox, sheep, or pig pituitaries and discuss its
separation from the luteinizing substance referred to by other workers.
More recently, however, Evans [1938] appears to regard the luteinizing
substance as identical with his 'antagonist'.

*Experiments on inhibition of gonadotrophic activity by luteinizing
extracts.* In the present experiments extracts were mixed before sub-
cutaneous injection; the gonadotrophic extract, which was inhibited by
combination with another, was generally pregnant mare serum extract.
Two very active extracts, PMS16 and PMS18 were used: Tables VI and
VII show the ovarian response produced by these substances in total doses

of 0·5–2·5 mg., in the usual 5-day test on groups of 10 rats. The remaining data in the tables show that the addition of extract of pig, ox, or sheep hypophysis substantially reduced the size of the ovaries caused by a given dose of pregnant mare serum. The amounts of hypophyseal extracts were generally such that, given alone, they would produce a relatively small

Table VI. *Effect of luteinizing hypophyseal extracts on response to pregnant mare serum extract PMS16*

Test no.	Pregnant mare serum extract total dose	Hypophyseal extract total dose	Ovarian weight mg.	No. of rats
R	PMS16 2·5 mg.	—	189	20
R	PMS16 1·0 mg.	—	78	15
R	PMS16 0·5 mg.	—	34	10
18	PMS16 2·5 mg.	Pig AP51 25 mg.	77	10
19	PMS16 1·0 mg.	,, ,,	36	10
20	PMS16 0·5 mg.	,, ,,	25	10
24	PMS16 2·5 mg.	Ox AP52D1 25 mg.	38	10
22	PMS16 1·0 mg.	Sheep AP57D 12·5 mg.	52	10

increase in ovarian weight. When PMS16 was given in combination with a standard 25 mg. dose of pig hypophyseal extract AP51 (Table VI), a partial inhibition of the gonadotrophic activity of the pregnant mare serum extract appeared to take place; the ovarian weights remained roughly proportionate to the dose of PMS16, but were greatly reduced as compared with the weights when the same doses of serum were given alone. Test 32 (Table VII) shows also an inhibiting action by the same pig hypophyseal

Table VII. *Effect of luteinizing hypophyseal extracts on response to pregnant mare serum extract PMS18*

Test no.	Pregnant mare serum extract total dose	Hypophyseal extract total dose	Ovarian weight mg.	No. of rats
33	PMS18 2·5 mg.	—	243	20
38	,, ,,	Ox AP52D1 5 mg.	139	10
36	,, ,,	Ox AP52D1 10 mg.	100	10
37	,, ,,	Ox AP52D1 25 mg.	90	10
25	PMS18 10·0 mg.	Ox AP52D1 25 mg.	119	10
29	PMS18 2·5 mg.	Ox AP52C 25 mg.	163	10
30	PMS18 1·0 mg.	—	103	10
58	,, ,,	Ox AP52C 25 mg.	62	10
31	,, ,,	Ox AP52D2 25 mg.	77	10
32	,, ,,	Pig AP51 25 mg.	62	10

extract on the second pregnant mare serum extract PMS18. In the converse experiment (Table VII), in which a standard 2·5 mg. dose of serum PMS18 was combined with increasing doses of luteinizing extract of ox pituitary gland, the extent by which the ovarian weight increase was reduced was not directly proportionate to the amount of inhibiting ox

hypophyseal extract (tests 33, 36, 37, 38), although the latter caused rather more inhibition than the smaller ones.

In a further experiment (test 25), the response to 10 mg. of PMS18 (4 times the dose giving 243 mg. ovaries) was greatly reduced by 25 mg. of the same ox pituitary extract, the ovaries weighing 149 mg. This extract also reduced the ovary weight in combination with 2·5 mg. of PMS16 (test 24, Table VI).

The remaining tests listed in Tables VI and VII provide further examples of inhibition of the normal ovarian weight increases, when pregnant mare serum was injected in combination with other extracts. Test 22 (Table VI) shows inhibition of PMS16 by sheep hypophyseal extract. Tests 29, 58, and 31 (Table VII) show that extracts (C fractions) of ox pituitary gland, which themselves lack any perceptible gonadotrophic activity, may partially inhibit the action of pregnant mare serum extract [cf. Freud, 1937b], though not to the same extent as corresponding quantities of the gonadotrophic luteinizing fraction of the same extract. As might be expected, horse pituitary extract AP41D had no inhibitory action on PMS18; the ovaries showed a summation of the response produced by the two active extracts.

In addition to these experiments, two group tests were carried out in which urine of pregnancy extract was combined with pregnant mare serum extract. In the first of these 1 mg. of UP10, which would normally produce ovaries weighing about 40 mg., was combined with 1 mg. of PMS18, and although the ovaries, after the combined injections, showed no summation of weight increase, they averaged 93 mg., not significantly different from the ovaries produced by the same dose of PMS18 given alone (Table VII). In the second test 10 mg. of UP10 (10 times the dose giving the maximum ovarian weight for this type of extract) was added to 2·5 mg. of PMS18 and the resulting weight of the ovaries was 155 mg., as against 243 mg. for the same dose of pregnant mare serum extract given alone, so it may be concluded that inhibition had definitely taken place. Evidence was also obtained that the gonadotrophic activity of horse hypophyseal extract AP61B, which in a dose of 2·5 mg. produces ovaries averaging 58 mg., could be inhibited by adding pig hypophyseal extract, the solutions being mixed and injected subcutaneously. Twenty-five milligrams of pig hypophyseal extract added to the above dose of AP61B (test 59) produced ovaries weighing only 31 mg.

Evans, Simpson, Tolksdorf, and Jensen [1939] report somewhat similar experiments, in which the response of immature female rats to pregnant mare serum was reduced when a purified pituitary gland fraction was simultaneously injected; the solutions were mixed before intra-peritoneal injection. With a standard dose of pregnant mare serum extract increasing

amounts of the pituitary gland fraction caused, on the whole, increasing inhibition of the ovarian response.

No attempt will be made to offer a detailed explanation of these experiments on inhibiting gonadotrophic activity; the reactions between the extracts probably depend, in most cases, on the individual action of each of the two gonadotrophic substances on the ovary, the final results being affected by the relative rates of action of the two substances. The tests with the inert fractions of hypophyseal extracts suggest, however, that a more or less direct action between the pregnant mare serum and the inhibiting extract may also be possible.

SUMMARY

Groups of immature female rats have been used for testing various gonadotrophic extracts. A study has been made of the extent to which the effectiveness of given amounts can be modified by (1) varying the number of injections, (2) delaying absorption by the addition of inert substances, and (3) combining two different extracts.

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THE EFFECT OF ROUTE OF ADMINISTRATION ON THE MULTIPLE ACTIVITIES OF TESTOSTERONE AND METHYL TESTOSTERONE IN DIFFERENT SPECIES

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THE variation which is encountered in the absolute and relative activities of endocrinologically active substances, according to the method of administration, has been the object of much study. An investigation into the activities of certain androgens having strong physiological activity other than androgenic brought to light instances in which the various activities of a substance seemed to be exerted to a different degree, not only according to the species of test animal, but also according to the route of administration. Changes of the latter type, in which the same test animal is considered, present peculiar difficulties. Unless relatively minor differences in action are sought, such as that between the response of the prostate and of the seminal vesicles in male rats, the various recognized tests for androgenic, progestational, and other activities are not, with the partial exception of those with the rat, adapted to an investigation of this kind. Thus, the rabbit, used for assessing progestational activity, is not readily usable for other assays, such as those of androgenic potency.

The various activities of testosterone when given by injection to rats and capons have been very fully discussed in the literature, while Klein and Parkes [1937] first reported that it has progestational activity in rabbits. Methyl testosterone [Ruzicka, Goldberg, and Rosenberg, 1935] has also received attention, and has been shown by Deanesly and Parkes [1936 a] to be appreciably more active than testosterone on the prostate and seminal vesicles of castrated rats, but only about ⅓th as active in the capon comb test. In the test for progestational activity as described by McPhail [1934] methyl testosterone was found by Klein and Parkes [1937] to be more active than testosterone in producing progestational proliferation. According to Miescher and Tschopp [1938] the methyl derivative retains its activity, when given by mouth to castrated rats, much better than does testosterone.

In the present investigation, the relative effectiveness of oral and parenteral administration of these two compounds has been examined in a variety of tests with the same or different species of animals.

METHODS AND RESULTS

Androgenic Activity in Capons

Tests on the combs of Brown Leghorn capons were carried out as described by Emmens [1939b], activity being determined by reference to control groups of birds receiving androsterone, and to standard curves. The relative activities of testosterone when given by inunction, by injection, and by mouth have already been described [Emmens, 1939a]. In Table I these are compared with the corresponding data for methyl testosterone, which is found to be about as active as testosterone when they are given by mouth, but less active by other routes of administration.

Androgenic Activity in Castrated Rats

The technique of assay on rats was similar to that described by Callow and Deanesly [1935]; the rats were adult when used, and had rested for 2 months after castration. All assays in this series of experiments were carried out with groups of 5 rats per dose (Table II), with a 10-day test period, injections or feedings being made once daily in propylene glycol. Comparing the oral activity of testosterone with that found previously by injection in this laboratory [Deanesly and Parkes, 1936a], we find that 0·6 mg. total given in a daily volume of 0·2 ml. of oil gave a prostate weight

Table I. *Approximate total amounts (μg.) of testosterone and methyl testosterone which produce a 5 mm. increase in length plus height of the combs of Brown Leghorn capons when given by different routes. (Figures corrected for changes in the sensitivity of the birds)*

Substance	By injection	By mouth	By inunction	$\left(\dfrac{\text{Dose by mouth}}{\text{Dose by injection}}\right)$
Testosterone	85	2500	2·1	30
Methyl testosterone	365	3000	4·7	8

Table II. *Activity of testosterone and methyl testosterone in propylene glycol on the adult castrated rat. Each assay was carried out on a group of 5 rats*

Date of assay	Substance	Route of administration	Total dose mg.	Daily volume ml.	Average weight of: Prostate mg.	Seminal vesicles mg.
23/1/39	Testosterone	Oral	5	0·1	38	8·4
,,	Methyl testosterone	Injected	2	0·5	65	26
,,	,,	,,	10	,,	145	84
,,	,,	Oral	2	0·1	30	7·2
24/6/39	,,	,,	5	,,	65	23
11/11/38	,,	,,	10	,,	89	32
,,	,,	Tablet implantation	3·8	—	409	293

of 33 mg. and a seminal vesicle weight of 10 mg., similar figures now being found for 5 mg. total given orally. Since injecting daily with 0·2 ml. volumes is a relatively ineffective method, at any rate with oil [Deanesly and Parkes, 1936 b], the oral activity of testosterone is clearly low, of the order of $\frac{1}{10}$th or less of its activity by injection. With methyl testosterone, on the other hand, 5 mg. given orally are equal in effect to 2 mg. given by injection, with the relatively efficient daily volume of 0·5 ml. The oral activity of methyl testosterone is therefore of the order of $\frac{2}{5}$ths its activity when given by an effective injection technique. By injection, methyl testosterone is clearly of greater potency than testosterone. Rats receiving methyl testosterone in the form of implanted tablets, an average of 3·8 mg. of which was absorbed over a 10-day test period, had a prostate weight of 409 mg. and a seminal vesicle weight of 293 mg. The great effectiveness of this method of administration is again demonstrated. Nevertheless, in view of the previous findings in this laboratory, that an average of 1·5 mg. of testosterone absorbed over 10 days produced a prostate plus seminal vesicle weight of 877 mg. [Deanesly and Parkes, 1937], it is probable that methyl testosterone fails to maintain its superiority over testosterone when given by this method.

Metrotrophic Activity in Spayed Rats

For these tests, immature female albino rats were spayed at 40–60 g. body-weight, and rested for 2–3 weeks before being used. The final body-weight of groups at the end of the tests was between 80 and 140 g. The test period was 5 days, using 5 rats per group. An injection or feeding of the test material in oily solution was given once each day, and killing took place on the 6th day, 24 hours after the last injection. The uteri were dissected and fixed in aqueous Bouin's solution for 24 hours, and weighed after a similar period in 70% alcohol. The results are given in Table III. Testosterone and methyl testosterone have about equal metrotrophic activity when injected in 0·2 ml. of oil per day.

The dose/response relationships for methyl testosterone given orally or by injection are seen to be approximately linear when the response is plotted against the logarithm of the dose (Fig. 1). The slope of the line for oral administration is a little flatter than that for injection. Methyl testosterone is between 4 and 8 times as potent by injection in 0·2 ml. of oil daily as when given orally, depending on the level of response at which comparison is made, and about 20 times as potent by injection in 0·5 ml. of oil per day. It seems improbable that the volume of oil in which the oral dose is given affects its potency, especially as a further experiment showed that 2·5 mg. of methyl testosterone ingested during a 5 day test period in 5% gum arabic in the drinking water had approximately the same

effect as when given by once-daily feedings (Fig. 1). On the assumptio[n] that dose/response lines for testosterone would have the same slope a[s] those for methyl testosterone, it may be calculated from the tests wit[h] this compound that it has between 8 and 16 times the potency by injectio[n] as by mouth, when a volume of 0·2 ml. per day is employed, and doubtles[s] a still greater relative potency if a larger volume of oil is injected.

Table III. *Activity of testosterone and methyl testosterone in oil on the uteru[s] of spayed rats, 5 rats per group*

Date of assay	Substance	Route	Total dose mg.	Daily volume ml.	Body-weight at death g.	Average uterine weight mg.
15/10/38	Testosterone	Injected	5	0·2	80	36
,,	Controls	—	—	—	76	15
24/10/38	Testosterone	Injected	10	0·2	109	54
,,	,,	Oral	,,	,,	98	21
,,	Controls	—	—	—	101	16
4/11/38	Methyl testosterone	Injected	10	0·2	117	59
,,	,,	Oral	,,	,,	136	35
,,	Controls	—	—	—	121	16
23/1/39	Methyl testosterone	Injected	2	0·2	116	36
,,	,,	,,	4	,,	130	39
,,	,,	,,	8	,,	112	55
,,	,,	,,	16	,,	107	65
,,	,,	Oral	4	,,	,,	23
,,	,,	,,	8	,,	103	32
,,	,,	,,	16	,,	107	37
,,	,,	,,	32	,,	113	45
,,	Controls (10 rats)	—	—	—	116	17
6/2/39	Methyl testosterone	Injected	4	0·5	140	65
,,	,,	Oral	2·5	In drinking water	111	20

The action of these compounds on the uterus of the rat is partly myo-trophic, but there appears to be some progestational stimulation of the endometrium, which shows a few glandular crypts, a high columnar endo-thelium with nuclei raised from the basement membrane, and a somewhat enlarged lumen.

Metrotrophic Activity in the Immature Rabbit

In these tests, Dutch, Himalayan, or Albino immature female rabbits were given one injection on each of 3 consecutive days, and killed on the 4th day. The uterus was dissected out, fixed for 24 hours in Bouin's solution, and weighed after a similar period in 70% alcohol, as in the rat tests. The control material was collected over a period, from Dutch and

Himalayan rabbits only. The uterus of the immature female remains below 200 mg. until a body-weight of 1 kg. is approached, and groups of 9 or 10 females below 800 g. have an average uterine weight of considerably less than this. Table IV gives the data for these tests, all injections and feedings being in 1 ml. per day of propylene glycol, with one exception,
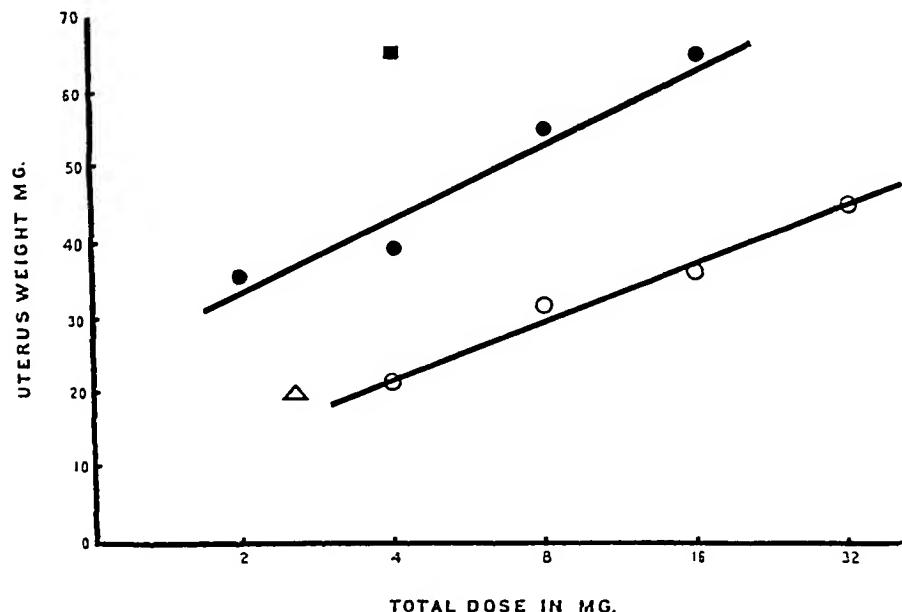
Fig. 1. The metrotrophic activity of methyl testosterone in spayed rats, five per group.
(See Table III.)

● = by injection in 0·2 ml. of oil per day.
■ = by injection in 0·5 ml. of oil per day.
○ = by mouth in 0·2 ml. of oil per day.
△ = by mouth in the drinking water.

marked in the table, in which oil was used. The results with these tests cannot be called satisfactory, as individual variation in response is excessive. Testosterone, in a dose of 15 mg., produced an apparently significant response by injection, but not by mouth, while the responses with methyl testosterone are altogether too variable for any definite conclusions to be drawn. The positive response given by injection on 18/7/38 is annulled by that of 7/8/38, in which no increase in uterine weight was demonstrable. It is perhaps possible to conclude that both testosterone and methyl testosterone are significantly more active in this test by injection than by mouth, as simultaneous tests by the two routes have always shown a greater response in the case of the injected group.

Table IV. *Activity of testosterone and methyl testosterone in propylene glyco on the uterus of immature female rabbits*

Date of assay	Substance	Route	Total dose mg.	No. of rabbits	Average body-weight g.	Average uterine weight mg.	Range
11/7/38	Testosterone	Oral	15	2	650	81	41–120
,,	,, (in oil)	Injected	,,	,,	635	167	162–171
18/7/38	,,	Oral	,,	5	434	113	20–337
,,	,,	Injected	,,	,,	508	190	133–251
30/8/38	,,	Oral	9	,,	422	42	21–57
,,	,,	Injected	,,	,,	466	54	31–70
29/6/38	Methyl testosterone	Oral	15	2	325	157	102–212
18/7/38	,,	,,	,,	,,	565	87	73–101
,,	,,	Injected	,,	3	760	442	200–767
7/8/38	,,	Oral	,,	5	414	52	45–65
,,	,,	Injected	,,	,,	374	63	45–73
—	Controls	—	—	9	331	37	28–51
—	,,	—	—	10	544	53	27–137
—	,,	—	—	9	714	72	24–172

Progestational Activity in Rabbits

By the test for progestational activity as described by McPhail [1934] or by a modification in which only three daily injections of the test substance are given, both compounds show activity by mouth and by injection (Table V).

The responses have been graded from $\frac{1}{2}$ to 4, whole numbers corresponding with McPhail's proliferation indices of + to ++++, and an average figure has been taken for each group. With one exception in which oil was used, marked in the table, the solvent has been propylene glycol. Dutch rabbits have mainly been used, but the use of other breeds does not seem to have much influence on response.

When given by mouth, testosterone appears to be less than $\frac{1}{4}$ as active as when injected, while methyl testosterone is only slightly less active orally than by injection. When they are compared by a 3-day test, methyl testosterone has about twice the activity of testosterone. A group response of over 2·6 has not been obtained in these tests, and it is noteworthy that 20 mg. doses of methyl testosterone given in the assay dated 3/9/38 did not elicit any greater response than doses of 10 mg. The alteration in volume from the usual 1·0 to 0·5 ml. in those particular tests can hardly have affected the oral potency, but may possibly have lowered the potency by injection. It seems possible that full proliferation is not obtainable with these substances, and in no individual case has a higher index than 3 been obtained.

Table V. *The progestational activity of testosterone and methyl testosterone by the McPhail test, when given orally or by injection*

Date of assay	Sub-stance	Route	Total dose mg.	Daily volume ml.	No. of daily injec-tions	No. of rabbits	Breed	Average re-sponse
18/7/38	Testosterone	Oral	15	1·0	3	3	Dutch	1·2
24/10/38	,,	,,	,,	,,	,,	4	Himalayan	0·5
2/11/38	,,	,,	,,	0·5	,,	5	Dutch	0·8
10/5/39	,,	,,	,,	1·0	,,	,,	,,	·0·7
,,	,,	,,	30	,,	,,	,,	,,	0·9
,,	,,	Injected	15	,,	,,	,,	,,	1·5
,,	,,	,,	30	,,	,,	,,	,,	2·4
26/6/38	Methyl testosterone	Oral	15	,,	,,	4	,,	1·9
2/7/38	,, (in oil)	,,	,,	,,	,,	3	Himalayan	2·0
,,	,,	,,	10	,,	5	,,	Lop	2·3
3/9/38	,,	,,	5	,,	,,	5	Dutch	0·7
,,	,,	,,	10	,,	,,	,,	,,	2·2
,,	,,	,,	20	0·5	,,	,,	,,	,,
7/8/38	,,	Injected	15	1·0	3	4	,,	2·3
3/9/38	,,	,,	5	,,	5	5	,,	1·1
,,	,,	,,	10	,,	,,	,,	,,	2·6
,,	,,	,,	20	0·5	,,	,,	,,	,,

Differences in response between species, and absolute differences in activity according to route of administration

The difference in the relative potency of testosterone and methyl testosterone when tested on capons and on rats is a striking instance of the variation encountered in the assay of such compounds when different species, as well as different methods of administration, are used. Whereas in capons methyl testosterone is less potent than, or only just equal in potency to, testosterone, according to the method of administration, it is more potent than the latter in all tests with castrated male rats. Similar but less striking differences in the activities of other androgens have already been discussed by Emmens [1938].

The greater relative oral effectiveness of methyl testosterone, when compared with that of testosterone in the castrated male rat test, might be due to a relatively more effective absorption from the gut, but its low metrotrophic activity when given orally to ovariectomized female rats seems to dispose of this possibility. In the rat, therefore, methyl testosterone, when given by mouth, as compared with injection, is a relatively better androgen than metrotroph. We have thus an example of a difference in relative multiple activity due to change in the route of administration.

The high oral effectiveness of methyl testosterone in causing progest: tional proliferation in rabbits cannot be put down to such an absolu: change in the ratio between its several potencies, dependent upon tl method of administration, because its androgenic or other activities i rabbits have not been fully ascertained from our data.

We already know that a compound may have different potencies rel: tive to another compound, when tested by different methods on the sam or different species, and may vary in potency on a given organ of a give species according to how it is administered. In the case of methyl testo sterone we have also been able to show that it may exhibit, according t the method of administration, difference between the ratios of its activitie on different organs of the same species of animal.

The reason for the last difference is not apparent, but it is possible tha the various activities of such substances as methyl testosterone are due not to the multiple activity of the one molecule, but to the separate proper ties of various breakdown products. If so, the relative intensity of th various multiple activities would be determined by the proportions i which the breakdown or transformation products were formed, which i turn might well be influenced by oral as compared with subcutaneou: administration and by the species of animal receiving the parent substance

SUMMARY

1. The androgenic and gynaecogenic activities of testosterone and methyl testosterone have been investigated in capons, rats, and rabbits by inunction and by oral and parenteral administration.

2. Testosterone is much less active by mouth than by injection in tests for androgenic activity in the capon and castrated male rat, in metro-trophic tests on spayed rats or immature rabbits, and in tests for pro-gesterone-like activity in rabbits.

3. Methyl testosterone, however, is almost as active by mouth as by injection in causing progestational proliferation in rabbits, and has a rela-tively greater activity by mouth in the other tests than has testosterone. When given orally instead of by injection to rats, its androgenic activity decreases much less than its power of causing an increase in uterine weight.

4. Methyl testosterone is more potent than testosterone as an androgen in rats, but less potent in capons, and more potent than testosterone in the progestational tests whichever route of administration is considered. The two substances are about equally active in causing uterine growth in spayed rats and immature rabbits.

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SOME BIOLOGICAL PROPERTIES OF ANHYDRO-HYDROXY-PROGESTERONE (ETHINYL TESTOSTERONE)

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THE capacity to evoke progestational changes in the uterine endometrium was originally thought to be specific to progesterone, the hormone of the corpus luteum, a substance which is difficult to obtain in quantity, and which is inactive by mouth. Klein and Parkes [1937], however, found that testosterone and androstenedione have a slight progesterone-like activity, while the methyl and ethyl derivatives of certain of the androgens, notably methyl testosterone, have an activity about $\frac{1}{20}$th that of progesterone itself. Since methyl testosterone differs from progesterone only in having the groups —OH and —CH$_3$ in position 17 instead of —H and —COCH$_3$ this result, though disproving the supposed complete specificity of progesterone, emphasized the importance of the short side chain in conferring specific activity on progesterone. Recently, an important advance has been made by the addition of an ethinyl group to testosterone, giving a compound which differs from progesterone in the absence of H$_2$ and O from the side chain, and in the presence of —OH instead of —H in the 17 position [Inhoffen, Logemann, Hohlweg, and Serini, 1938; Ruzicka, Hofmann, and Meldahl, 1938]. The progestational activity of this compound, anhydro-hydroxy-progesterone, 17-ethinyl-testosterone, or Δ^4-pregnen-in-17-ol-3-one, was first described by Inhoffen and Hohlweg [1938] who found that it has $\frac{1}{3}$rd of the activity of progesterone, giving a positive result in immature female rabbits in a total dose of 2 mg. However, the most important property possessed by anhydro-hydroxy-progesterone is its oral activity, 4 mg. giving a positive result in Inhoffen and Hohlweg's tests. In capons, no comb growth was obtained with 2 mg. Further confirmatory observations followed by Ruzicka, *et al.* [1938], who found that 4–6 mg. by injection or 10 mg. given orally were effective in their tests. Courrier and Jost [1939] have now shown that it is capable of maintaining pregnancy in spayed rabbits and that it is androgenic in castrated rats and in the chick comb test, both in doses of 10–20 mg. per day for 10 days. Clinical effects of the substance have been recorded by Salmon, Walter, and Geist [1939] and by Zondek and Rozin [1939].

The introduction of what seems to amount to an orally active form of

progesterone must be regarded as an important discovery, though the incidental biological properties of the compound must not be forgotten. We have already made a preliminary report on an investigation into these [Emmens and Parkes, 1939], and this is described in detail below.

METHODS AND RESULTS

Androgenic Activity on Capons

Tests on induction of growth of the capon comb were carried out as described by Emmens [1939 b], using a 3-day test period, with one dose per day. Comb growth is expressed as the increase in length plus height, as measured on the day after the last injection or treatment. After preliminary tests, groups of 4 or 5 capons were used for a simultaneous comparison of the activity of anhydro-hydroxy-progesterone when given by injection, by inunction, and by mouth. Owing to its low solubility in other media which can be used for injection, the compound was injected and fed to the birds in propylene glycol at a concentration of 2 mg. per ml., 10 mg. being given per day in each case. Inunction was made in nut oil at a concentration of 0·5 mg. per ml., 0·05 mg. being given per day. The results are shown in Table I.

Table I. *Androgenic activity of anhydro-hydroxy-progesterone in the capon comb-growth test*

Route	Total dose mg.	Solvent		Daily volume mL.	No. of birds	Average response mm.
By injection	30	Propylene glycol		5	4	4·6
By mouth	,,	,,	,,	,,	,,	3·5
By inunction	0·15	Nut oil		0·1	5	2·2

Although a very weak androgen, with about $\frac{1}{500}$th the potency of testosterone by injection, anhydro-hydroxy-progesterone is seen to be as active by mouth as by injection. By inunction it has about 100 times its potency by injection or mouth, agreeing in this respect with other androgens which have been found to give corresponding ratios between 40 and 250 [Emmens, 1939 a].

Progestational Activity in Rabbits

Progestational activity was assessed by the McPhail test [1934], using immature female rabbits primed with oestrone, or by a modification of it in which 3 instead of 5 daily injections of the test substance are given. Positive responses were graded from 1 to 4, whole numbers corresponding with McPhail's indices of + to ++++. The results, given in Table II,

confirm those of previous workers in showing the high activity of the orally administered substance.

Table II. *Progestational activity of anhydro-hydroxy-progesterone on the rabbit uterus*

Date of assay	Route of administration	No. of rabbits	Total dose mg.	No. of administrations	Daily vol. ml.	Solvent	Breed of rabbit	Average response	Range
3/5/38	Subcutaneous	5	5	5	1	Oil	Lop	2·4	2–3
,,	Oral	1	,,	,,	,,	Propylene glycol	,,	2·0	—
23/5/38	,,	3	,,	,,	,,	,,	,,	2·5	2–3
29/5/38	,,	5	,,	,,	,,	15% alcohol	Himalayan	0·4	0–1·5
24/10/38	Subcutaneous	,,	9	3	0·5	Propylene glycol	Himalayan+ Albino	1·8	1–2·5
,,	Oral	,,	,,	,,	,,	,,	,,	1·3	1–1·5
26/3/39	Subcutaneous	,,	5	5	,,	,,	Dutch	2·4	1–3
,,	Oral	4	,,	,,	,,	,,	,,	2·4	2–3·5

By the McPhail technique, progesterone in a dose of 0·5 mg. (0·5 I.U.), whether injected over 3 or 5 days, gives a response of between 2 and 3. Anhydro-hydroxy-progesterone evokes this response with a total dose of 5 mg. when given over 5 days in propylene glycol either orally or by injection, but appears to be less active in a 3-day test. From Table II the following conclusions may be drawn:

(a) Anhydro-hydroxy-progesterone is as active by mouth, when given in propylene glycol solution, as it is by subcutaneous injection in propylene glycol or in oil.

(b) The compound is about $\frac{1}{10}$th as active as progesterone compared by injection, but indefinitely more active than progesterone by mouth.

(c) The oral activity of anhydro-hydroxy-progesterone may be affected by the solvent, since a total of 5 mg. given by mouth in a 15% alcohol solution elicited a response of only 0·4, 3 of the 5 test animals showing no significant proliferation.

Activity on the Uterus and Vagina of Spayed Rats

The rats used in these tests were spayed 3 weeks before use, the body-weight of groups at the end of the tests averaging from 98 to 119 g. Tests were made by oral and parenteral administration by 5 injections or feedings, once daily for 5 days, with killing on the 6th day, 24 hours after the last injection. Vaginal smears were taken at killing, and the uteri were dissected out and fixed in aqueous Bouin's solution for 24 hours, and

weighed on a torsion balance after a similar period in 70% alcohol. In another series of tests, both uteri and vaginae were fixed, sectioned at 7μ, and stained with Erlich's haematoxylin and counterstained with eosin.

Preliminary tests showed that the activity of anhydro-hydroxy-progesterone in increasing the weight of the spayed rat's uterus is about the same by mouth as by injection. Eight groups of 5 rats per group were therefore simultaneously given 4 different total doses, by mouth and by subcutaneous injection in propylene glycol, the size of the doses being in geometrical progression. The volume per animal for injection was 1 ml. daily, and for oral administration 0·2 or 0·4 ml. daily. The results are shown in Table III.

Table III. *Activity of anhydro-hydroxy-progesterone in propylene glycol on the uterus and vagina of the spayed rat*

Route of administration	Total dose mg.	Average body weight g.	Average uterine weight mg.	No. of rats	No. with open vagina	Vaginal smears
Injected	0·5	113	23	5	2	—ve
,,	1·0	98	21	,,	4	,,
,,	2·0	119	28	,,	3	1+ve
,,	4·0	113	35	,,	5	4+ve
Oral	0·5	115	21	,,	0	—ve
,,	1·0	94	32	4	1	,,
,,	2·0	114	33	5	3	,,
,,	4·0	115	28	,,	1	,, (some cornification)
Controls	—	116	17	10	0	—ve

The relation of dose to response in this series of tests was very unsatisfactory, but the results serve to show that the activity on the uterus is about the same by either route, as far as the increase in weight is concerned. The activity on the vagina, however, both in causing vaginal opening and cornification, was less by mouth than by injection. Dosage by injection appears from this series to be approximately 4 times as effective as by mouth in both cases. In a second series of tests, made primarily for the purposes of a histological investigation, vaginal cornification was produced in some of the rats receiving doses of 20 mg. given orally. The reaction of the rat uterus to progesterone has been described by Selye, Collip, and Thomson [1935]. They distinguish two phases, the 'first progestational reaction', characterized by a slightly enlarged uterus, a well-developed sub-mucosa, with a poorly developed muscular wall, and a uterine epithelium with many mitotic figures and no cilia or secretion; and the 'second progestational reaction', in which the greatly enlarged uterus has a ciliated epithelium with higher secretory cells. The mucosa

forms many folds which project into the lumen, and the muscular w
is well developed.

The reaction of the uteri of ovariectomized rats primed with 2 dai
doses of 10 μg. of oestrone and injected for 5 days with 2 mg. or 5 m
total of progesterone was first studied. Uteri weighing between 36 a
40 mg. resulted from the lower dose of progesterone, and between
and 60 mg. from the higher dose. They were apparently characteristic
the 'first progestational reaction' described above, but muscle-layers we
quite well developed and few mitoses were seen (Plate I, Fig. 1). Eve
with a total dose of 5 mg. of progesterone, the epithelium was very litt
or not at all folded and was not ciliated. A total dose of 20 mg. of anhydr
hydroxy-progesterone (without previous treatment with oestrone) whe
given by mouth, caused an increase in uterine weight to between 37 an
59 mg. and the histological appearance was broadly similar to those treate
with progesterone, but with a larger lumen, folds in the epithelium, an
a little less submucosal tissue. Total doses of 10 mg., given orally, produce
uteri between 29 and 52 mg. with an appearance very like that given b
progesterone, but the epithelium was again somewhat more folded. Wit
2 mg. the uteri weighed 23–32 mg. and presented a similar appearance
there being less development in all layers (Plate I, Figs. 2–3). By injec
tion, with the same range of doses, the compound produced very simila
effects (Plate II, Figs. 4 and 5), but the uterine lumen was smaller in animal
receiving a total of 20 mg. than in those given 20 mg. orally. However
this was probably due to the fact that the uteri of these particular rat
did not increase so much in weight as did those receiving oral treatment
being between 32 and 39 mg. In uteri of approximately the same weight
regardless of the quantity of anhydro-hydroxy-progesterone producing it
a comparison of the oral and parental activities shows that, in general,
a larger lumen and a smaller amount of sub-mucosal tissue is present in the
injected animals than in those receiving the compound by mouth (compare
Plate I, Fig. 3, and Plate II, Figs. 4 and 5). It seems probable, as we
already suggested [Emmens and Parkes, 1939], that the progestational
effect of anhydro-hydroxy-progesterone is less complicated by its oestro-
genic or other properties when it is administered orally than when it is
given by injection, since its oestrogenic action on the vagina appears to
be lower under the former conditions.

Oestrogenic Activity in Spayed Rats and Mice

From Table III, the dose required to produce 50% of cornified vaginal
smears by injection into rats is seen to be about 3 mg., but, as the substance
was given over a period of 5 days, a smaller dose would probably be effec-
tive in a typical Allen-Doisy test, using 2 or 4 injections over 2 days.

Vaginal cornification produced by oral administration in such a rat is shown in Plate II, Fig. 6, and appears to be histologically typical.

An approximate estimate of the amount required to produce cornification in spayed mice was made by injecting or feeding series of mice with graded doses, two animals on each dose given by each route, by two daily administrations in propylene glycol. All mice receiving more than 0·8 mg. by injection gave positive responses, all receiving less gave negative responses, while the two mice receiving 0·8 mg. gave one a positive and the other a negative response. The dose required to produce 50% of cornified smears is therefore approximately 0·8 mg. by this technique. Mice receiving up to 1·6 mg. orally gave negative responses, and it was not possible to give a higher oral dose than this, without either feeding a suspension of crystals or giving a toxic dose of the solvent.

Summary

1. In the capon comb-growth test, anhydro-hydroxy-progesterone shows weak androgenic activity, but is equally active by mouth and by injection, having about $\frac{1}{600}$th the activity of injected testosterone.

2. Anhydro-hydroxy-progesterone is equally active by mouth and by injection in producing progestational proliferation in rabbits when given in propylene glycol, having about $\frac{1}{10}$th the potency of injected progesterone.

3. Anhydro-hydroxy-progesterone produces growth of the uterus of the spayed rat, without previous treatment with oestrogen. Its effects are very similar to those produced by progesterone in the uterus already primed with oestrone. It appears to be more effectively progestational when given orally, although of equal potency by mouth and by injection in causing an increase in uterine weight.

4. Cornification of the vagina of the spayed rat or mouse and vaginal opening in the spayed rat are caused by anhydro-hydroxy-progesterone. In these respects it is more active by injection than by mouth.

5. In addition to its high oral activity in the different tests, anhydro-hydroxy-progesterone seems to be the only compound so far described which has progesterone-like, metrotrophic, androgenic, and oestrogenic properties.

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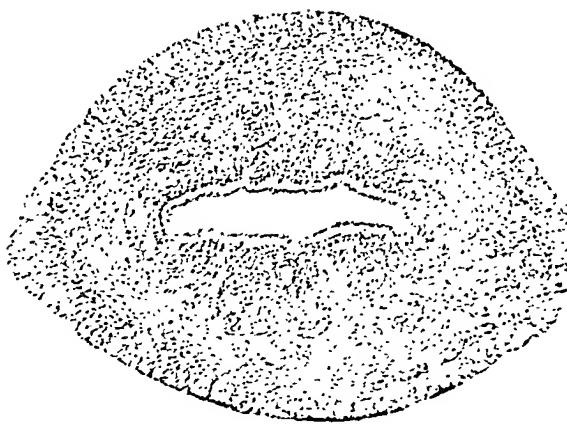
FIG. 1. Uterus (weighing 60 mg.) of spayed rat after oestrone followed by 5 mg. total of injected progesterone. × 57.
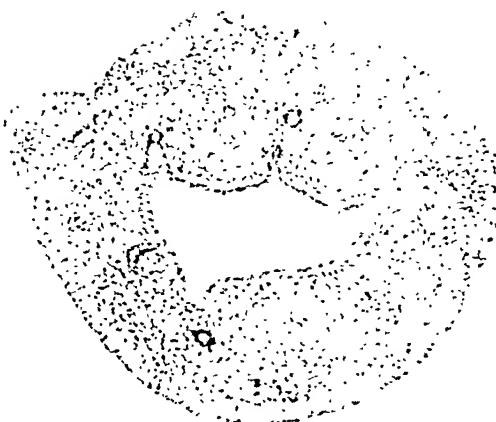
FIG. 2. Uterus (weighing 59 mg.) of spayed rat after 20 mg. of anhydro-hydroxy-progesterone given orally. ×57.

FIG. 3. Uterus (weighing 31 mg.) of spayed rat after 2 mg. of anhydro-hydroxy-progesterone given orally. × 57.

FIG. 4. Uterus (weighing 37 mg.) of spayed rat after 20 mg. of injected anhydro-hydroxy-progesterone. × 57.
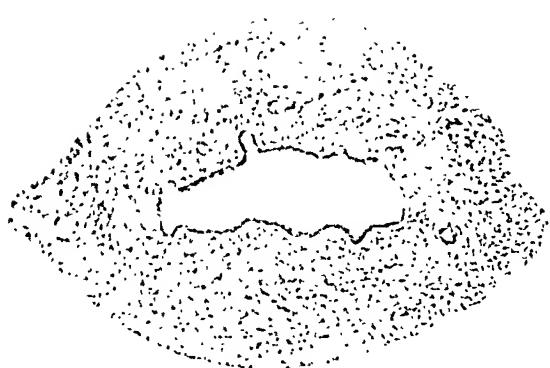
FIG. 5. Uterus (weighing 29 mg.) of spayed rat after 2 mg. of injected anhydro-hydroxy-progesterone. × 57.

FIG. 6. Vagina of spayed rat showing cornification caused by anhydro-hydroxy-progesterone given orally. × 225.

ANTERIOR PITUITARY FRACTIONS AND CARBOHYDRATE METABOLISM

I. THE PREPARATION AND PROPERTIES OF DIABETOGENIC EXTRACTS

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IN previous communications [Young, 1936, 1937c, 1938a] it has been shown that none of the following anterior pituitary fractions is diabetogenic *per se*, when tested by the daily administration of large amounts to an intact dog: prolactin, thyrotropic hormone, glycotropic substance, gonadotropic substance, the latter being assayed by its ability to cause ovulation in the oestrous rabbit. More recently, Houssay and Biasotti [1938] tested a number of anterior lobe fractions for ability to induce a diabetic condition in dogs from which the pituitary gland and most of the pancreas had been removed. The following substances were found to have no significant effect: prolactin, adrenotropic hormone, follicle-stimulating hormone, luteinizing hormone.

The present communication records efforts to purify diabetogenic extracts of ox anterior lobe by fractionation with ammonium sulphate at 0° C. The properties of crude and of purified diabetogenic extracts were examined, and some observations on their biological action also recorded.

METHODS

Biological

Diabetogenic activity of anterior lobe extracts was determined by the daily injection of large doses into normal young male dogs for 1–3 weeks. Injections were made intraperitoneally, and the amount administered daily increased successively on the 4th, 7th, 10th, &c., day of treatment. The details of the method of administration have previously been described [Young, 1938b]. A typical test is illustrated in Fig. 1.

Thyrotropic activity was assayed by ability to increase the weight of the thyroid gland of the immature guinea-pig, according to the method described by Rowlands and Parkes [1934]. The International Standard preparation of thyrotropic hormone, in terms of which the International Unit of activity is to be defined, not being available, it was impossible to express the results in actual International Units. By the decision recorded, however, by the Third International Conference on the

Standardization of Hormones held at Geneva by the Health Organization of the League of Nations in August 1938,[1] the international unit of thyrotropic activity which is to be defined in terms of the International Standard when that becomes available, should be closely similar in magnitude
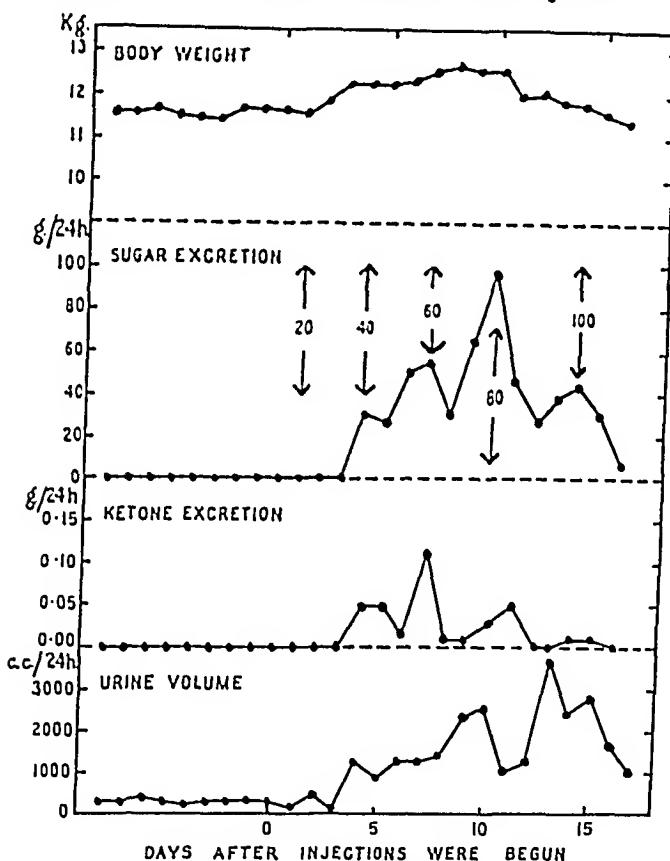
FIG. 1. Dog 57. Data relating to the diabetogenic action of the pseudoglobulin fraction. Daily injections of extract were begun on day 1. The figures on the arrows indicate the number of g. of fresh tissue equivalent to the dose of pseudoglobulin fraction injected daily, from that day until the day indicated by the next arrow.

to that proposed by Rowlands and Parkes [1934]. The results of assays in the present investigations have therefore been expressed in terms of the temporary Rowlands-Parkes units, such a unit being defined as that amount of active material which, when administered subcutaneously on each of 5 successive days to a 200-g. female guinea-pig, doubles the weight of the thyroid glands. No substantial inaccuracy should be caused by assuming that the units so used will be practically identical with the International Unit.

Gonadotropic activity was determined on the basis of ability to cause

ovulation in the oestrous rabbit. The results were expressed in terms of the units defined by Hill, Parkes, and White [1934], viz. the ovulation-producing activity required to cause ovulation in 50% of a group of not less than 10 oestrous rabbits, the extract being administered as a single intravenous injection. I am indebted to Dr. I. W. Rowlands for carrying out a number of these assays. It should be noted that as yet no International Standard Unit for the gonadotropic activity of pituitary substances has been defined.

Glycotropic activity of pituitary extracts was assessed on the basis of ability to induce chronic insensitivity to the hypoglycaemic action of insulin, the latter being administered to a previously treated fasting rabbit. This method has been described in previous communications [Young, 1936, 1938 c, d].

Growth-promoting activity was determined either on the hypophysectomized rat (male or female), or on the normal female rat. I am greatly indebted to Dr. E. Bülbring of the Pharmacology Department, University of Oxford, for carrying out the assays on the hypophysectomized animals. For this purpose groups of 5 hypophysectomized rats of 80–100 g. weight, which had previously not increased in weight over a period of 7 days, were each given 7 equal daily injections of extract, and the average weight-increase of the group over this period was determined. Assays on normal female rats were carried out by determining the mean increase in weight of a group of 10 rats averaging initially about 150 g. body-weight, during a period of 14 daily injections of extract.

Chemical

Preparation of extracts. Fresh ox pituitary glands were used in these investigations, the glands being frozen and transported from the slaughter-house in solid carbon dioxide. The anterior lobes were dissected free while the glands were still frozen. All subsequent procedures were carried out at 0° C., or as near to this temperature as was possible.

A crude alkaline extract was prepared by grinding the minced anterior lobes with saline at pH 8·5; the details of this procedure have already been described [Young, 1938 b]. In the present investigation two successive extractions were effected, in both cases the volume of saline for extraction being 2 c.c. for each gramme of fresh anterior lobe tissue. The combined pH 8·5 extracts were adjusted to pH 5–5·5, and the insoluble material centrifuged off, extracted at pH 8·5, and reprecipitated at pH 5–5·5 three or more times [cf. Young, 1938 a]. The combined pH 5·5 supernatant fluids were treated according to the scheme depicted in Fig. 2; the final fractions were: (a) material precipitated by one-third saturation with ammonium sulphate, and completely insoluble in aqueous solution from

pH 8·5 extract of ox anterior lobes
| adjust pH to 5–5·5.

PRECIPITATE

= prolactin fraction. Reprecipitated a number of times at pH 5 5·5 and material soluble at this pH added to main soluble fraction (1).

SOLUBLE FRACTION (1) (vol. = x c.c.)

— { Add $\frac{x}{2}$ c.c. of sat. ammon. sulphate solution.

PRECIPITATE

Washed twice with one-third sat. ammon. sulphate sol. and washings added to soluble fraction (2). Dissolved at pH 8·5 and reprecipitated twice by one-third saturation with ammonium sulphate. Soluble fractions added to main soluble fraction (2). Precipitate dissolved at pH 8·5 and dialysed until free from salts; any material remaining in solution after prolonged dialysis discarded. Precipitate dissolved at pH 8·5 = EUGLOBULIN FRACTION.

SOLUBLE FRACTION (2) (vol. = $x+y$ c.c.)

— { Add $\frac{x+y}{3}$ c.c. sat. ammon. sulphate solution.

PRECIPITATE

Washed twice with half-sat. ammon. sulphate sol. and washings added to soluble fraction (3). Dissolved at pH 8·5 and reprecipitated twice by half saturation with ammonium sulphate. Soluble fractions added to main soluble fraction (3). Precipitate dissolved at pH 8·5 and dialysed until free from salts; any precipitate formed as the result of prolonged dialysis discarded.

Solution = PSEUDOGLOBULIN FRACTION.

SOLUBLE FRACTION (3)

— { Stir in solid ammon. sulphate until fully saturated.

PRECIPITATE

Washed twice with saturated ammon. sulphate solution and washings discarded. Precipitate dissolved in water and dialysed until free from salts; any precipitate formed as the result of prolonged dialysis discarded.

Solution = ALBUMIN FRACTION.

SOLUBLE FRACTION (4)
Discarded.

FIG. 2. Fractionation of fresh ox anterior lobe tissue. All these processes were carried out in the cold-room at 0° C. as far as possible. Precipitates were isolated by a combination of decantation and filtration where possible, rather than by centrifuging. A special apparatus, designed by Mr. E. A. Woollett, was used for the automatic filtration of the large volumes obtained (see Appendix). About 500 g. of fresh ox anterior lobe tissue was found to be a suitable amount to work up at one time.

which inorganic salts had been removed by prolonged dialysis ('euglobulin fraction'); (*b*) material which was soluble in one-third saturated ammonium sulphate solution, but was precipitated when the concentration of ammonium sulphate was raised to one-half saturation, and remained in solution when all inorganic salts were removed by prolonged dialysis ('pseudoglobulin fraction'); (*c*) material which was soluble in one-half saturated ammonium sulphate solution, but was precipitated by full saturation with ammonium sulphate, and remained in solution after prolonged dialysis ('albumin fraction'). In some preliminary experiments the 'pseudoglobulin' and 'euglobulin' fractions were precipitated together by half-saturation with ammonium sulphate, and the two fractions then separated by prolonged dialysis, the euglobulin fraction precipitating when inorganic salts had been removed, while the pseudoglobulin fraction stayed in solution under these conditions. No essential difference was found between fractions prepared by this method and those prepared by the method outlined in Fig. 2, but the latter method was ultimately adopted as being more likely to give a complete separation of the two fractions.

In some instances, where storage at 0° C. for some time was necessary, the solutions of the final fractions were Seitz-filtered, but this process, with its attendant losses, was avoided wherever possible. No preservatives were added to the solutions, which were stored in the cold-room at just above 0° C. and used as soon as possible after preparation. It was found desirable to store the solutions without freezing, as irreversible coagulation of the euglobulin and pseudoglobulin fractions was prone to occur if the solution was frozen. The solutions were generally stored without the addition of salts, 0·9% of NaCl being added just before injection. It was sometimes found that a part of the euglobulin fraction had precipitated from solution in water at pH 8·5 when such a solution had been stored some days without added NaCl, but this precipitate readily redissolved when 0·9% NaCl was added.

The final volume of the solutions was so adjusted that 1 c.c. of water-clear solution contained material from about 2 g. of fresh anterior lobe tissue.

The percentage of solid material in the dialysed solutions of the different fractions was determined by drying known volumes to constant weight at 100° C.; the average results are given in Table I.

Chemical estimations. Blood-sugar was estimated on 0·1 c.c. of blood by the Hagedorn-Jensen method.

Urine sugar was estimated by Benedict's method.

Glycogen was determined by a modification of the method of Evans, Tsai, and Young [1931].

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Table I. *Amount of non-dialysable solid material in protein fractions from ox anterior lobe*

		Mg. solid equivalent to 1 g. of fresh tissue			
		Globulin fraction			Albumin fraction
		Total	Euglobulin	Pseudoglobulin	
Maximum	. .	14·1	5·3	7·9	14·4
Minimum	. .	5·0	1·4	2·2	8·4
Average	. .	7·6	3·15	4·45	11·3

The results are based on 8 experiments

Urine ketones were estimated by the gravimetric Denigès-van Slyke method, or when the amount was too small to be estimated by the gravimetric method, by an approximate assessment on the basis of colour tests.

RESULTS

Diabetogenic Activity

The results are summarized in Table II, while the findings for dog 57, which received treatment with the pseudoglobulin fraction, are illustrated in Fig. 1. The results show that the euglobulin and albumin fractions exhibited no diabetogenic activity under the conditions of these tests, while the globulin fraction and its pseudoglobulin constituent possessed diabetogenic action in the intact dog. Nevertheless, the diabetogenic

Table II. *Tests for diabetogenic action on normal dogs*

Fraction	Number of preparation	Dose injected daily; g. equiv. of fresh tissue		Number of dog	Number of daily injections given	Number of days on which glycosuria was observed	Maximum glycosuria g./day
		Min.	Max.				
Whole	B₁, C₁, & D₁	40	80	54	7	7	94
globulin	D₆	15	50	72	9	4	31
Euglo-	D₁	20	100	58	13	0	—
bulin	D₄	10	30	9	0	0	—
	D₇	20	60	76	8	0	—
	D₈	20	60	78	9	0	—
Pseudo-	D₁ & D₂	20	100	57	14	13	97
globulin	D₄	10	30	63	9	5	6
	D₇	20	60	77	8	5	17
	D₈	20	60	79	9	1	6
Albumin	B₂, C₂, & D₂	40	80	55	6	0	—
	D₁	20	80	61	13	0	—
	D₄	10	30	65	9	0	—
	D₆	15	50	74	9	0	—

activity of the pseudoglobulin fraction was not as great as might be expected; in fact, one of the tests, namely, that on dog 79, was barely positive. It seems, however, that dog 79 was somewhat abnormal and possessed an unusually high renal threshold. Subsequent treatment of this animal with a crude anterior lobe extract failed to induce a sustained glycosuria, although the blood-sugar level was in the region of 0·170%. Failure of normal dogs to respond to the diabetogenic action of a crude ox anterior lobe is, in our experience, very rare [cf. also Houssay and Biasotti, 1938]. Of a series of 35 normal dogs treated with a crude saline extract of ox anterior lobe tissue, only one failed to exhibit glycosuria; this animal was the only pregnant bitch in our series [Young, 1938 *b*].

In some instances dogs which had failed to exhibit· diabetes when treated with the euglobulin or albumin fractions, subsequently received daily injections of a crude anterior lobe extract. All these animals demonstrated their responsiveness to the diabetogenic action of the extract by exhibiting glycosuria and hyperglycaemia.

Thyrotropic Activity

Average results are given in Table III, which also includes for the purpose of comparison mean results for the initial crude alkaline extract of anterior lobe and for the pH 5–5·5 soluble fraction of this crude extract. There was considerable difference in the results for different preparations

Table III. *Thyrotropic activities of the different fraction expressed in Rowland-Parkes Units*

		Crude alkaline extract	pH 5–5·5 soluble fraction	Globulin			Albumin
				Total	Euglobulin	Pseudoglobulin	
Units per g.	Maximum	13·2	11·2	9·2	1·8	8·6	2·0
equivalent of	Minimum	7·2	5·9	1·1	0·1	1·0	0·4
fresh tissue	Average	*9·9*	*8·5*	*4·4*	*0·5*	*1·0*	*1·7*
Units per mg.	Maximum	—	—	1·0	0·3	1·5	0·18
dry substance	Minimum	—	—	0·2	0·0	0·2	0·03
in fraction	Average	—	*0·25*	*0·5*	*0·15*	*0·3*	*0·13*

The results are based on 4 experiments.

of the same type of fraction, but it appears that the globulin and pseudoglobulin fractions, which are diabetogenic, possess more thyrotropic activity than the other fractions under consideration. On the average the pseudoglobulin fraction contains about 40% of the thyrotropic substance present in the original crude alkaline extract, whereas the albumin fraction contains less than 20% and the euglobulin fraction about 5% of the original amount.

Gonadotropic Activity

Only a few assays for gonadotropic activity were carried out, but the results of these resembled those for thyrotropic activity in indicating greater activity for the pseudoglobulin than for the euglobulin fraction. Typical results are given in Table IV.

Table IV. *Gonadotropic activities of the different fractions*

Hill-Parkes-White units	Globulin fraction			Albumin fraction
	Total	Euglobulin	Pseudoglobulin	
Per g. equiv. of fresh tissue	34·6	0·6	34·0	35·0
Per mg. of dry substance in fraction	5·2	0·2	9·5	4·2

The albumin fraction contains as much gonadotropic substance as do the total globulin or the pseudoglobulin fractions, and it is clear that the latter fractions do not possess diabetogenic activity by virtue of their content of gonadotropic substance.

Glycotropic Activity

Typical results are illustrated in Fig. 3, from which it will be seen that there is no very great difference between the activities of the different fractions. The pseudoglobulin fraction is, however, slightly more active than the others on the whole, the albumin fraction and the euglobulin fraction being about equally active, but a little less active than the pseudoglobulin fraction.

Growth-promoting Activity

The results, which are summarized in Table V, indicate that greater growth-promoting activity is found with the pseudoglobulin fraction than with the other comparable fractions tested. The albumin fraction was found to possess little or no growth-promoting activity in our tests.

Table V. *Growth-promoting activity of the different fractions*

Extract	Hypophysectomized rats		Normal female rats	
	Daily dose. G. equiv. of fresh tissue	Growth g./7 days	Daily dose. G. equiv. of fresh tissue	Growth above that of controls g./14 days
Crude extract	—	—	0·125	+35
pH 5–5·5 sol. material	0·2	+8	—	—
Whole globulin fraction	0·5	+8	—	—
Euglobulin fraction	2·0	+8	2·0	−2
Pseudoglobulin fraction	2·0	+19	2·0	+20
Albumin fraction	1·0	−6	2·0	+4
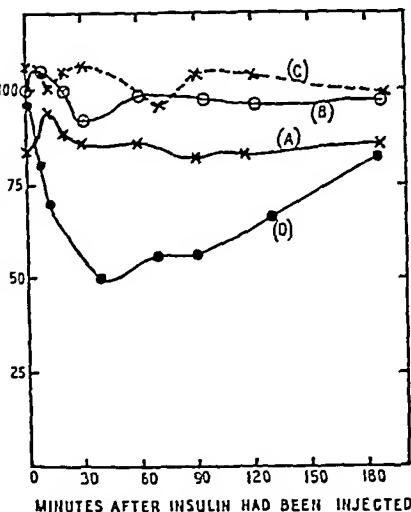
FIG. 3. Glycotropic activity of the different fractions as indicated by the glycaemic response to 2 units of crystalline insulin administered intravenously to fasting rabbits which had previously been treated with the fraction.

Curve A. Rabbit which had received 2 injections each equivalent to 5 g. of fresh tissue of the pseudoglobulin fraction.

Curve B. Rabbit which had received similar treatment with the same dose of euglobulin fraction.

Curve C. Rabbit which had received similar treatment with the same dose of albumin fraction.

Curve D. Control experiment. No previous treatment.
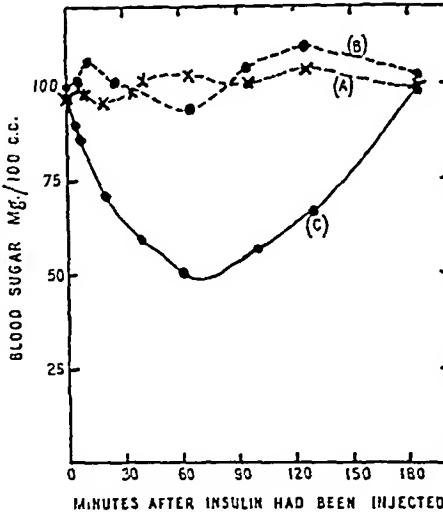
FIG. 4. Glycotropic activity of a crude pituitary extract which had been heated at pH 10 on a boiling-water bath.

Curve A. Response to 2 units of crystalline insulin of fasting rabbit which had received two injections of a crude pituitary extract, each equivalent to 0·8 g. of fresh anterior lobe tissue.

Curve B. Response to 2 units of crystalline insulin of fasting rabbit which had received similar treatment with the same dose of extract which had been heated at pH 10 on a boiling-water bath for 1 hour.

Curve C. Control experiment. No previous treatment.

The Heat-lability of the Diabetogenic Substance

An anterior lobe extract which has been heated at pH 10 on a boiling-water bath for 1 hour still retains a large proportion of its glycotropic activity [Collip, 1938; Young, 1938 e]. Neufeld and Collip [1938] showed that such a heated extract was without diabetogenic action when injected into a normal dog, thus providing evidence confirmatory of the non-identity of the diabetogenic and glycotropic substances [Young, 1936, 1937 c, 1938 c, d]. In two experiments on dogs we also observed a complete lack of diabetogenic action of a crude anterior lobe extract heated thus at pH 10, although, as illustrated in Fig. 4, no obvious diminution in glycotropic activity occurred as the result of this treatment.

Houssay, and subsequently the present author, have stressed the importance of obtaining absolutely fresh pituitary glands in a frozen condition,

and of preparing the extracts in the cold-room, in order to demonstrate
the diabetogenic activity of anterior lobe extracts. In Table VI is recorded
the result of an experiment in which a crude alkaline extract of ox anterior
lobe was divided into two portions, one being allowed to remain at room
temperature for 24 hours, the other being preserved at 0° C. Subsequently,
that portion of the extract which had been allowed to become 'stale' at

Table VI. *Experiment on the stability at room temperature of a crude
diabetogenic extract*

Injection g. equivalent of fresh tissue/day	Dog 85—stale extract				Dog 86—fresh extract			
	Daily urine excretion				Daily urine excretion			
	Vol. c.c.	Sugar g.	Ketone g.	Body wt.	Vol. c.c.	Sugar g.	Ketone g.	Body wt.
0	620	0	0	12·20	570	0	0	12·50
0	630	0	0	11·90	1020	0	0	12·95
0	820	0	0	12·10	520	0	0	13·25
0	820	0	0	11·45	850	0	0	13·15
0	530	0	0	11·65	760	0	0	13·40
5	740	0	0	11·70	1020	0	0	13·40
5	620	0	0	11·45	850	0	0	13·40
5	1520	0	0	11·00	880	15·8	0·01	13·45
10	790	0	0	10·90	1780	52·5	0·01	13·95
10	1680	0	0	11·00	2470	85·2	0·10	14·00
10	720	0	0	11·25	2520	63·0	0·05	13·90
15	740	0	0	11·00	2360	59·0	0·01	14·00
15	520	0	0	10·65	2920	58·4	0·05	14·85
15	520	0	0	11·15	3080	84·5	0·05	14·25

room temperature was found to be without diabetes-producing activity
(dog 85), while the other portion exerted the usual diabetogenic action
(dog 86). The thyrotropic activity of the extract which had remained at
room temperature for 24 hours was not in the least diminished, again
demonstrating that thyrotropic extracts are not *per se* diabetogenic.

Apart from indicating the instability of the diabetogenic substance
under some conditions, the experiments with the material heated at pH 10,
and that with the extract which had remained at room temperature for
some time, all constitute excellent control experiments, illustrating the
innocuous results of the daily intraperitoneal injection of large volumes of
extract into dogs. Although the dogs exhibited no sign of diabetes when
receiving injections of ill-treated extract, dog 86, to which was administered
a carefully preserved anterior lobe preparation, exhibited a clearly defined
diabetic condition.

Some Chemical Properties of the Different Fractions

Table VII shows the results of some simple tests on the various fractions.
It is interesting to note that the nitrogen content (Kjeldahl) of the

pseudoglobulin fractions appears to be somewhat higher than that for the other fractions. On the other hand, the Molisch test for carbohydrate residues is much stronger with albumin fraction than with either of the two constituents of the globulin fraction. All three fractions gave precipitates with trichloracetic acid, sulphosalycylic acid, and with $AgNO_3$.

Table VII. *Chemical properties of active fractions*

Test	Pseudoglobulin	Euglobulin	Albumin
Biuret . . .	++	++	++
Xanthoproteic . .	+	+	++
Millon . . .	+	+	++
Hopkins-Cole . .	++	++	?
Molisch . . .	?	?	++
Sulphur . . .	+	+	++
Phosphorus . .	?	?	trace
Nitrogen (Kjeldahl) .	16·2%	13·7%	14·4%

Solutions of the euglobulin and pseudoglobulin fractions are pale straw-coloured, but that of the albumin fraction is invariably dark red. The pigment in this fraction was identified by Dr. C. Rimington as methaemoglobin, but, as a crude extract of anterior lobe was found to contain oxy-haemoglobin and no detectable methaemoglobin, it appears that oxidation of the pigment must have occurred during the process of fractionation.

The Influence of Diabetogenic Extracts on Liver and Muscle Glycogen Contents and on Liver Fat Content in the Intact Dog

Houssay, Biasotti, and Dambrosi [1936] found that the average fasting liver and muscle glycogen contents of 6 dogs made diabetic by anterior lobe extract were 2·732% and 0·515% respectively, whereas the corresponding figures for 9 control animals were, respectively, 2·105% and 0·528%. These authors conclude that in dogs made diabetic by anterior lobe extract the liver glycogen level is increased, whereas the muscle glycogen content is little affected or slightly falls. Young [1937 b] found that both liver glycogen and muscle glycogen levels were abnormally high in intact rabbits which had been rendered insulin-resistant, but not diabetic, by treatment with anterior lobe extracts.

Table VIII gives liver glycogen and muscle glycogen data for 5 control and for 6 diabetic dogs. The liver glycogen figures are exceptionally variable (much more so than those of Houssay and his colleagues), and it cannot be assumed on the basis of these results that there is any significant difference between the average figures for liver glycogen content of these two groups, although the average value for the diabetic animals is slightly below that for the control group, and not above, as in the results of Houssay and his colleagues. Our figures for muscle glycogen indicate an average value which may be significantly lower for the diabetic animals, although

the difference is not statistically significant on the basis of our limited number of observations. It is surprising that the general level of glycogen in our animals is so much higher than that in the animals of Houssay *et al.*

Table VIII. *Data for glycogen and fat in untreated and treated fasting dogs*

No.	Liver glycogen %	Liver fat %	Muscle glycogen %	Liver wt. % body-wt.	Remarks
(a) Control dogs					
81	7·05	2·11	1·50	3·5	—
82	2·75	—	1·01	2·6	—
83	2·45	3·72	0·92	2·6	—
84	4·05	2·60	1·05	3·1	Bitch
93	8·55	—	1·12	2·8	—
Average	4·97	2·81	1·13± 0·099	2·9	
(b) Dogs made diabetic by crude anterior lobe extract					
16	1·40	7·80	—	—	—
75	4·70	—	0·50	—	—
78	2·75	3·83	0·98	—	—
86	1·58	7·91	0·85	5·3	—
87	4·58	—	0·84	5·5	—
88	6·45	—	1·15	5·6	—
Average	3·58	6·51	0·86± 0·106	5·5	
(c) Dogs which had become refractory to the diabetogenic action of pituitary extract					
37	4·00	—	0·75	5·2	Crude extract
72	9·90	—	2·25	—	Globulin fraction
74	4·50	—	1·17	—	Crude extract
77	3·05	—	0·65	—	Pseudoglobulin fraction
79	4·30	2·2	1·05	6·2	Crude extract
Average	5·15	—	1·17	—	

A few results for liver fat content [cf. Munoz, 1938] and for liver size are included in Table VIII. These figures show that both the liver fat content and liver size of the diabetic animals is increased. As the size of the liver appears to be greater in the diabetic animals, the total amount of glycogen in the liver is probably significantly greater than in the control dogs, although the proportion appears to be slightly less.

Observations on the liver and muscle glycogen contents of dogs which had developed refractoriness to the diabetogenic action of the extract after showing an initial response, indicated that these values were not significantly different from those for control animals (Table VIII). Animals in this condition are still relatively insensitive to the hypoglycaemic action of injected insulin [Young, 1939 b].

DISCUSSION

The results obtained in the present investigation show that diabetogenic activity is present in the globulin fraction of ox anterior lobe, but that

the albumin fraction shows no diabetogenic activity when administered daily to an intact dog. The diabetogenic activity of the globulin fraction is not found in the euglobulin constituent of this fraction, although some activity is found in pseudoglobulin fraction. In one experiment as little as 5·5 mg./kg. body-weight/day of the pseudoglobulin fraction was sufficient to initiate an intensely diabetic condition. On the whole, however, the diabetogenic activity of the pseudoglobulin fraction was disappointing, and it seems probable that losses must have occurred in the course of preparation in spite of the fact that all the processes were carried out in the cold-room at 0° C.

Except with dog 57, no serious attempt was made to convert the temporary diabetes induced by the treatment with the pseudoglobulin fraction into a permanent condition, as was previously found possible with a short period of daily injections of crude extract [Young, 1937 *a*, 1938 *d*]. In the experiment with dog 57 (Fig. 1) the supply of extract was unfortunately exhausted before this result could be achieved. There is no reason to doubt, however, that a permanently diabetic condition could be produced by prolonged treatment with such a purified extract. The conditions under which the temporary diabetes is converted into the permanent state have recently been discussed [Young, 1939 *b*].

The glycotropic and ketogenic [Gray and Young, 1939] activities of ox anterior lobe tissue are not present preferentially in the pseudoglobulin fraction, but are distributed among the euglobulin, pseudoglobulin, and albumin fractions. If the factors responsible for these effects are concerned in any way with the diabetogenic action, or are constituents of the diabetogenic principle, although not themselves diabetogenic, then the relatively low activity of the fraction in which diabetogenic activity was found would be explicable.

The thyrotropic hormone is concentrated to some extent in the pseudoglobulin fraction, and it has not proved possible in our experiments to obtain a diabetogenic preparation low in thyrotropic activity. In a previous investigation, however, it was shown that the daily administration of more than 300 Rowlands-Parkes units of thyrotropic hormone did not induce a diabetic condition in normal dogs, and in the present investigation the minimal effective doses of the pseudoglobulin fraction contained less than 150 units of thyrotropic substance; so that there is no reason to believe that the diabetogenic activity of our fractions is due to the thyrotropic hormone contained therein. Until a complete separation of the thyrotropic hormone and the diabetogenic principle can be effected, however, the question whether the thyrotropic hormone is a constituent of a diabetogenic complex must be left open.

The conclusion that the gonadotropic substance is not diabetogenic is

confirmed by the observation that the albumin fraction is as rich in gonado-tropic activity as the globulin fraction, or as the pseudoglobulin portion of the latter, but that the albumin fraction does not possess diabetogenic activity.

Our finding that diabetogenic activity may be associated with the growth-promoting substance of anterior lobe extracts is in agreement with previous observations [Evans, Meyer, Simpson, and Reichert, 1932; Baumann and Marine, 1932; Shipley and Long, 1938]. The possibility may be considered [cf. Young, 1939 a], in that connexion, that the nature of the response of an animal to the administration of a pituitary extract may be determined by the reaction of the islets of Langerhans of the pancreas. If the islets furnish sufficient insulin, the extract may induce nitrogen retention [cf. Mirsky and Swadesh, 1938; Harrison and Long, 1938; Mirsky, 1939] and exhibit a growth-promoting action, but, if in-sufficient insulin is available, then the same extract may exhibit a diabeto-genic action. The results of Mirsky [1939] concerning the influence of anterior lobe extracts on protein metabolism are of particular interest in this connexion. It should be mentioned that Marks and Young [1939] have shown that treatment of rats with crude anterior lobe extracts results in a substantial increase in the insulin content of the pancreas, without induction of a diabetic condition. It is of interest that our dogs usually increase in weight during the period of temporary diabetes induced by the daily administration of anterior lobe extract; although one must hesitate to assume that this is a true growth, it may be so, and it is possible that the growth-promoting and diabetogenic activities of the extract can be simultaneously manifest.

Whatever may be the case, it is clear that the rate of secretion of insulin by the pancreas will be of importance in determining whether or not an animal will respond to the diabetogenic action of an extract. Some strains of rat are peculiarly insensitive to the diabetogenic action of anterior lobe extracts [Long, 1937; Young, 1938 b], although, when a large part of the pancreas is removed from a rat of the same strain, the animal then becomes responsive to the action of the extract [Long, 1937; cf. also the experi-ments of Houssay, Biasotti, and Rietti, 1932, with partially depancreatized dogs]. The fact that the administration of anterior lobe extract increases the intensity of the diabetic condition of completely depancreatized dogs [Gerschman and Marenzi, 1935; Foglia, Gerschman, Marenzi, Munoz, and Rietti, 1937] indicates that the diabetogenic action of the extract is, at least in part, a process more fundamental than that of depressing the rate of secretion of insulin by the pancreas. Nevertheless, Houssay and Foglia [1936] have demonstrated that the pancreas of a dog made temporarily diabetic by the administration of anterior lobe extract is secreting less

ilin than the pancreas of a normal dog, and Campbell, Keenan, and
t [1939], confirmed by Marks and Young [1939], have shown that the
ncreas of such a dog contains only a small fraction of the normal
ount of insulin. This diminished availability of insulin may be a secon-
y effect of an extra-pancreatic action of the extract, causing a rise of
od-sugar which may then induce such a response in the islets of Langer-
ns as to deplete them of insulin; or it may be due to a direct action on
pancreas, depressing the production of insulin. It is, therefore, not
prising to find that an animal from which the major portion of the
ncreas has been ablated may develop a diabetic condition in response
the administration of an extract which is ineffective in a normal animal.
r instance, Houssay, Biasotti, and Rietti [1932], using non-diabetic,
rtially depancreatized dogs, found that in 1 experiment out of 8 a dia-
togenic action of dog muscle extract was demonstrable, while 1 case of
rcosuria was observed in 6 similar experiments with a crude extract of
ef thyroid gland. On the other hand, Houssay, Biasotti, Benedetto,
d Rietti [1932] found that diabetes was not produced in any of 19
rmal dogs treated with extracts of muscle, thyroid, posterior pituitary
be, or adrenaline. Similar negative control experiments have been
ported by other workers [cf. Young, 1938 b]. We have, therefore, pre-
rred to use the dog with pancreas intact for the purpose of testing the
ecific diabetogenic activity of anterior lobe fractions. Valuable results
ay also be obtained with partially depancreatized animals, but the final
ecision whether an extract is diabetogenic in the normal animal must be
ased on the result of a test with a normal animal.

Shipley and Long [1938] have obtained interesting and important
esults, using the partially depancreatized rats for the testing of the
iabetogenic activity of extracts. Their observations indicate an associa-
on between the ketogenic, diabetogenic, and growth-promoting activities
f anterior lobe extracts, but these authors have succeeded in preparing
actions which are diabetogenic under the conditions of their test, though
ossessing no detectable thyrotropic and gonadotropic activities and con-
aining very little prolactin. A direct comparison of the activities of the
ractions prepared by these authors and those described in the present
ommunication would be of great interest.

SUMMARY

1. By fractionation at 0° C. with ammonium sulphate, 'globulin',
pseudoglobulin', 'euglobulin', and 'albumin' fractions were prepared
rom ox anterior pituitary tissue. Only the 'globulin' and 'pseudoglobu-
in' fractions were found to be diabetogenic when injected daily into
normal dogs.

2. The diabetogenic fractions possessed thyrotropic, gonadotropic, and glycotropic activities, but preparations with such activities were not necessarily diabetogenic. The gonadotropic, thyrotropic, and glycotropic substances are therefore not diabetogenic *per se* in the intact dog. Extracts with diabetogenic activity were found to possess greater growth-promoting activity than non-diabetogenic fractions.

3. Some chemical properties of active fractions are described.

4. Dogs made temporarily diabetic by treatment with anterior lobe extracts were found to have less muscle glycogen, about the same amount of liver glycogen, and more liver fat, than do normal dogs under similar conditions.

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APPENDIX.—*A Simple Apparatus for the Automatic Filtration of Large Volumes of Liquid. Designed by E. A. Woollett*

The fractionation of diabetogenic anterior pituitary extracts requires the filtration of large volumes of liquid in the cold-room. A simple apparatus for automatically carrying out this operation has been designed by Mr. E. A. Woollett. It is described here as such apparatus may be of interest to those engaged in similar investigations.

The apparatus consists essentially of (*A*) a 10-litre round-bottomed flask; (*B*) a siphon tube; (*C*) a 6-in. funnel containing a fluted 27 cm. filter-paper— Whatman, No. 54; (*D*) a 'control' tube the lower end of which is *above* the level of the lower end of the siphon tube in funnel *C*, and the upper end of which is above the level of the liquid in flask *A*. The control tube *D* carries a two-way tap *F*. When the siphon tube *B* transfers liquid from the flask *A* into the funnel *C*, liquid is drawn up into the control tube *D* from the funnel, and an equilibrium state is reached, in which the atmospheric pressure in flask *A* is sufficiently reduced to prevent more liquid flowing through the siphon tube *B* into the funnel. The level of liquid in the funnel falls as filtration proceeds and finally drops below the level of the lower end of the control tube *D*. A bubble of air then runs up the control tube and causes a rise of atmospheric pressure in the flask *A*, so that liquid can then flow through the siphon tube into the funnel *C*. The flow stops when equilibrium has been reached. In this way the contents of flask *A* are slowly and completely transferred to the funnel *C* in a controlled manner.

Filtration can be most readily set in motion by connecting tube *G* with the air-space in the flask *A* by means of suitable manipulation of the tap *F*, and applying sufficient pressure to drive liquid through the siphon into the funnel. Immediately liquid begins to flow, the tap *F* is brought to its normal position and the control tube begins to operate. It is advisable to have the lower ends of the siphon and control tubes well down into the funnel *C*.

It is, of course, essential that the lower end of siphon tube *B* should always be below that of control tube *D*, in order that the liquid column in the siphon tube shall remain intact.
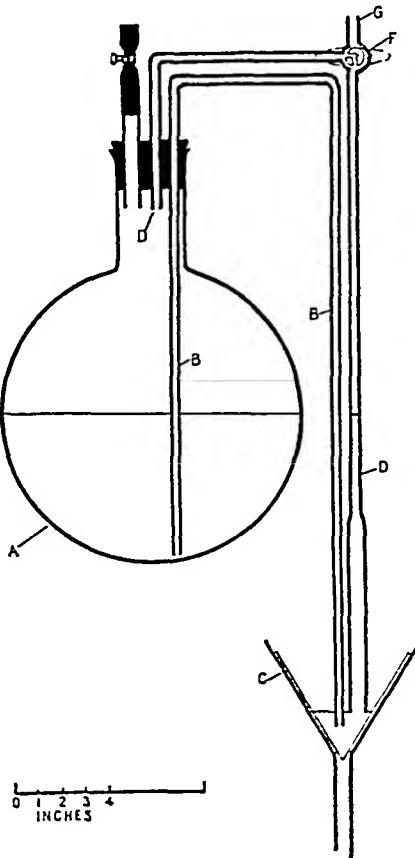
URINARY GONADOTROPHIC EXTRACTS AND ANAPHYLAXIS *IN VITRO*

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UNDER suitable conditions the anaphylactic response of isolated smooth muscle of the guinea-pig affords an easy, extremely sensitive, and highly specific *in vitro* test for the demonstration of antigens and antibodies. The method has been found to be valuable in the work here reported, on account of the sensitivity of the tests and the precision of the response, for it was found that antibodies could only be demonstrated with difficulty when the more usual *in vitro* methods were employed. Precipitins in the sera of rabbits which had received prolonged courses of injection of a urinary gonadotrophin (Pregnyl) have previously been described [van den Ende, 1939], and the same antisera have been used for the passive sensitization of guinea-pigs. Results of the tests for sensitivity of these guinea-pigs to a variety of antigens are recorded in this communication. Not only can the presence of antibodies in the antisera be demonstrated by anaphylaxis *in vitro*, but by comparison of the results obtained with gonadotrophic extracts on the one hand, and with known antigens from the same species on the other, an analysis of the antigenic complexity of the urinary gonadotrophins can be made.

The method is, however, not suitable for the demonstration of antibodies in antisera produced in any species of animal. Thus, antibodies prepared in the horse, goat, or ox are incapable of producing passive sensitization of guinea-pigs; presumably on account of the inability of the guinea-pig's tissues to fix the antibody molecules as found in the serum from these species [Hartley 1937, 1938].

The anaphylactic response to hormones in the whole animal has previously been studied by Smith [1934], using follicular fluids as antigens, and by Bernstein, Kirsner, and Turner [1938], using insulin. Smith concluded that species specificity could be demonstrated, and that follicular fluid was not auto-antigenic as was previously claimed by Lyons and Van de Carr [1930]. Bernstein *et al.*, on the other hand, succeeded in eliciting the symptoms of anaphylactic shock, using crystalline insulin as antigen. This is in agreement with the demonstration by Lewis [1937], using the *in vitro* technique, that insulin is a complete antigen, immunologically free of any species characteristics.

More recently Bischoff and Lyons [1939] have studied the immuno-logical properties of mammotrophic preparations. They conclude that mammotrophic extracts prepared according to the method described by Lyons [1937] from beef and sheep pituitaries are antigenically indistinguish-able as determined by the techniques employed for active and passive anaphylaxis, complement fixation and precipitation, as well as Dale and Arthus reactions. No species specificity could be demonstrated in the extracts. Their results cannot, however, be regarded as providing definite evidence of hormone specificity. The drastic treatment to which the pituitaries were subjected may have been enough to modify pituitary antigens other than the hormone sufficiently to mask any natural specific differences, which, owing to the close species relationship of the pituitaries used, might in any case have been very slight.

Precipitation tests have hitherto failed to demonstrate hormone-specific antibodies in antigonadotrophic sera. These experiments on anaphylaxis *in vitro* were not only undertaken as affording another qualitative test for the presence of hormone-specific antibodies, but the method suggested itself also as possibly providing a simple and rapid test for the diagnosis of pregnancy, if such hormone-specificity could be shown.

METHODS

Virgin female guinea-pigs weighing 225–50 g. were passively sensitized by the intraperitoneal injection of 1·0 to 5·0 c.c. of rabbit anti-Pregnyl serum. The antisera were actively antigonadotrophic, and were supplied by rabbits which had received daily subcutaneous injections of Pregnyl for 3 months or more.

Forty-eight hours after injection of the antiserum the guinea-pigs were stunned by a blow on the head and exsanguinated. The dissected uterine strips were suspended in Locke's solution according to the method de-scribed by Dale [1913]. One strip was tested for sensitivity to the homo-logous antigen (Pregnyl), and the other tested at the same time with one of the other antigens to be compared. These included the purified urinary gonadotrophins issued with the proprietary names Physex, Gonan, Prolan, Follutein, and an extract prepared from the urine of normal males by a method identical to that employed in the manufacture of Pregnyl (M.U.G.). The same preparations were used in the study of precipitins in antigonado-trophic sera [van den Ende, 1939]. Use was also made of a crude extract (alcohol precipitated) from the urine of a pregnant woman (P.U.G.), nor-mal human serum, and a gonadotrophic preparation from the serum of a pregnant woman. The available gonadotrophic extracts of pituitary glands all contained sufficient posterior lobe principles (oxytocin) to make their use in experiments on the isolated plain muscle impracticable.

Several guinea-pigs were actively sensitized by a single intraperitoneal injection of urinary gonadotrophin, a latent interval of 3–4 weeks being allowed, and others were passively sensitized by injection of an anti-gonadotrophic serum, prepared in rabbits by prolonged injection of an extract of human pituitary.
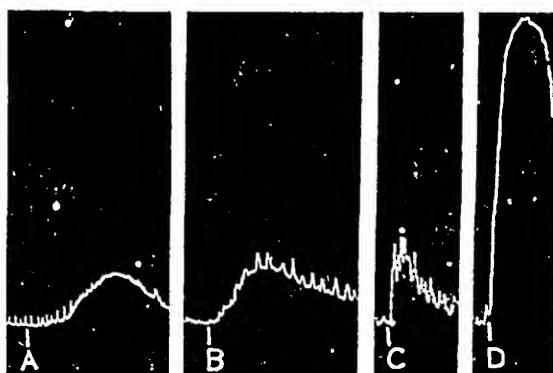
Fig. 1. Response of a normal guinea-pig uterus. At A. 10 mg. Pregnyl; B. 15 mg. Pregnyl; C. 20 mg. of a crude extract from the urine of a pregnant woman (P.U.G.); D. 20 mg. of a crude extract from the urine of a normal man (N.M.U.).

RESULTS

Some of the extracts (M.U.G., Pregnyl), when tested on normal uteri in the largest doses employed in this investigation, caused small, gradual, submaximal contractions, which could usually, without difficulty, be differentiated from true anaphylactic responses. In many experiments, single uterine strips were exposed to several different extracts in succession. In such cases, the amplitude of the rhythmic contractions of the uterus progressively increased and at the same time non-specific responses to urinary extracts became greater. Thus at the end of some experiments 5 to 10 mg. doses of Pregnyl or M.U.G. were found to elicit large, abrupt, non-specific contractions. These non-specific effects could, however, always be differentiated from anaphylactic responses, by the absence of specific desensitization in the former. Crude urinary extracts (P.U.G.) exhibited a greater non-specific activity in increasing the tone of normal smooth muscle, and for this reason doses larger than 20 mg. of such preparations could not be used (Fig. 1). Such small doses of a crude extract contain less than the minimal amount of antigenic material required to stimulate a genuine anaphylactic contraction, under the conditions of the tests.

Pregnyl in amounts of 5 to 10 mg. regularly gave rise to maximal anaphylactic contraction of sensitized uteri. Smaller doses (1–2 mg.) were equally effective, when more potent antisera, obtained after a period of

immunization of 6 months or more, were used for passive sensitization. Human serum (0·1–0·25 c.c.) elicited a small specific contraction of sensitized uterine muscle, but after desensitization to human serum such a uterus was still capable of maximal response to any one of the purified gonadotrophic urinary extracts: Prolan, Gonan, Follutein, Pregnyl.

The results of a typical experiment are shown in Fig. 2.
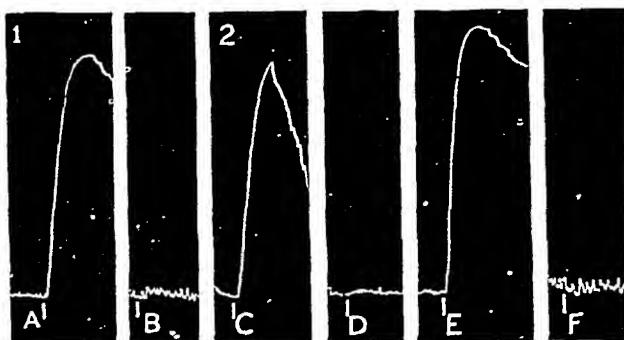
Fig. 2. Response of two uterine horns of a guinea-pig passively sensitized with anti-Pregnyl serum. 1st horn: At A. 5 mg. Follutein added to the bath; B. 5 mg. Follutein. 2nd horn: At C. 0·15 c.c. normal human serum; D. 0·15 c.c. normal serum—showing specific desensitization; E. 5 mg. Prolan; F. 5 mg. Prolan.

Anaphylaxis *in vitro* has established that Gonan and Prolan are similar to Pregnyl in antigenic composition. Not only do both of these extracts (Gonan or Prolan) contain antigens other than serum proteins capable of reacting specifically on smooth muscle sensitized to Pregnyl, but specific desensitization to either of them results also in the loss of sensitivity to Pregnyl, and vice versa (Fig. 4). Physex, on the other hand, differs from Pregnyl; a uterus passively sensitized with rabbit anti-Pregnyl serum responds with an anaphylactic contraction on contact with Physex, but retains a residual sensitivity to Pregnyl (Fig. 5). The experiments show clearly, therefore, that Prolan, Gonan, Follutein, Pregnyl, and Physex are complex antigens, for they all contain a species antigen, and the first four another common antigen, which appears to be absent from Physex. It is possible that there exists yet another antigen or antigenic grouping common to the series, on which the hormone activity depends. The hormone activity of an extract is, however, no indication of the power of that extract to elicit an anaphylactic response. Pregnyl, which is obtained from the urine of pregnant women, has 250 times the gonadotrophic activity of M.U.G., an extract prepared from the urine of normal men. Comparison of these two preparations is therefore of great interest. Fig. 3
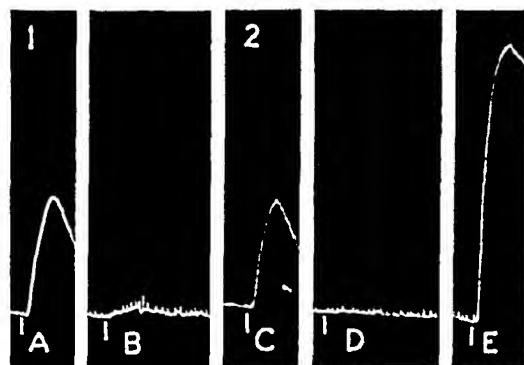
FIG. 3. Response of two uterine horns of a guinea-pig passively sensitized with anti-Pregnyl serum. 1st horn: At A. 2 mg. M.U.G. (male urinary extract); B. 5 mg. Pregnyl. 2nd horn: At C. 2 mg. Pregnyl; D. 5 mg. Pregnyl; E. 0·002 mg. Histamine.
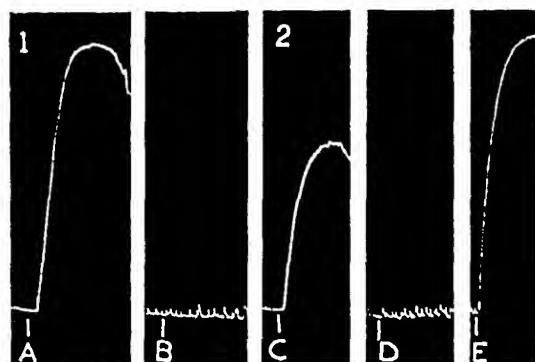
FIG. 4. Response of two uterine horns (1 and 2) of a guinea-pig passively sensitized with anti-Pregnyl serum. 1st horn: At A. 2 mg. Pregnyl; B. 4 mg. Pregnyl. 2nd horn: At C. 2 mg. Gonan; D. 4 mg. Pregnyl; E. 0·002 mg. Histamine.
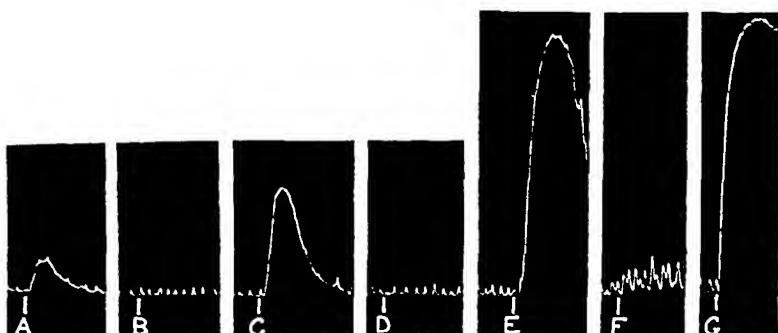
FIG. 5. Response of the uterus of a guinea-pig passively sensitized with anti-Pregnyl serum. At A. 0·1 c.c. human serum; B. 0·1 c.c. human serum; C. 10 mg. Physex; D. 10 mg. Physex; E. 10 mg. Pregnyl; F. 10 mg. Pregnyl; G. 0·002 mg. Histamine.

shows that the responses of two horns of the same sensitized uterus are approximately equal for the two preparations. Antigenically, therefore, these extracts are apparently identical, in spite of the wide difference in their gonadotrophic potencies.

The gonadotrophic principle from the urine of pregnancy is of placental origin, and its physiological effects differ from those of gonadotrophins of
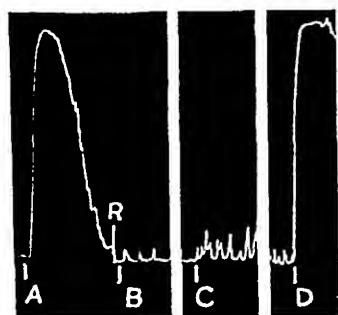
FIG. 6. Response of the uterus of a guinea-pig passively sensitized with anti-human pituitary serum. At A. 0·2 c.c. human serum (R. Ringer changed); B. 0·2 c.c. human serum; C. 10 mg. Pregnyl; D. 0·002 mg. Histamine.
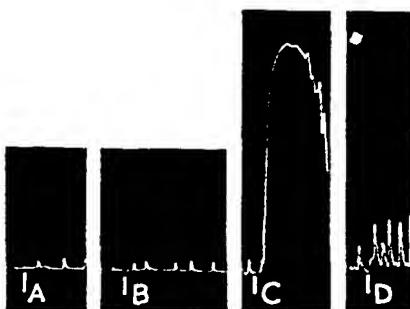
FIG. 8. Response of the uterus of a guinea-pig passively sensitized with anti-Pregnyl serum. At A. 0·25 c.c. human serum (a previous addition of 0·15 c.c. resulted in a small contraction only); B. 10 mg. of a gonadotrophic extract from the serum of a pregnant woman; C. 10 mg. Pregnyl; D. 10 mg. Pregnyl.

pituitary origin. Similar placental gonadotrophins occur in the blood-serum of pregnant women. The establishment of antigenic relationship, if any, between human serum and urinary gonadotrophins would therefore be of great interest. Results obtained give further evidence of the absence of hormone specificity of the antibodies in anti-Pregnyl sera. Thus, when a uterus passively sensitized with anti-Pregnyl serum has had its sensitivity to human serum abolished, not only does it fail to react to contact with an actively gonadotrophic extract from the serum of a pregnant woman, but such contact does not affect the anaphylactic response subsequently elicited by Pregnyl (Fig. 8).

Owing to the oxytocic properties of the pituitary gonadotrophins available, it was impracticable to test their anaphylactogenic power on smooth muscle *in vitro*. To compare pituitary gonadotrophins with those extracted from urine, therefore, recourse was made to an antigonado-trophic serum from rabbits injected with human pituitary extract. The smooth muscle of guinea-pigs passively sensitized by a single intra-peritoneal injection of 5 c.c. of this serum showed specific sensitivity to human serum proteins, but when it had been desensitized to these gave no reaction to urinary gonadotrophic extracts (Fig. 6).

In guinea-pigs actively sensitized to Pregnyl the degree of sensitivity to normal human serum proteins was greater than in the passively sensitized animals. In passively sensitized uteri one reaction to gonadotrophins resulted in complete desensitization of the smooth muscle to human serum as well. After active sensitization, on the other hand, a single response to
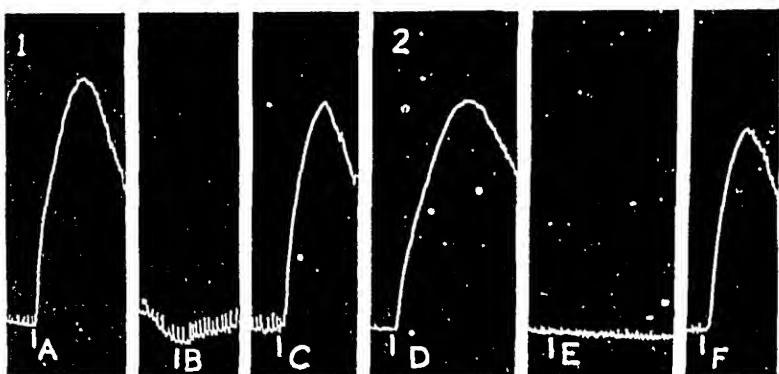
Fig. 7. Response of the two uterine horns of a guinea-pig actively sensitized with Pregnyl. 1st horn: At A. 5 mg. Pregnyl; B. 5 mg. Pregnyl; C. 0·1 c.c. human serum. 2nd horn: At D. 0·1 c.c. human serum; E. 0·1 c.c. human serum; F. 5 mg. Pregnyl.

a urinary gonadotrophin resulted in the loss of sensitivity to subsequent application of the gonadotrophin, but left sensitivity to human serum not perceptibly impaired; and, similarly, one response to serum resulted in loss of sensitivity to further contact with serum, while urinary gonadotrophin was still capable of eliciting a typical anaphylactic response (Fig. 7). The meaning of this difference between the actively and passively sensitized plain muscle, in respect of desensitization to human serum, is considered in the Discussion.

DISCUSSION

By anaphylactic methods it has been possible to confirm the results previously obtained with precipitin tests on antigonadotrophic sera. Antiserum prepared in rabbits against one preparation of human urinary gonadotrophin (Pregnyl) is capable of inducing sensitivity in the guinea-pig, as a result of which a specific anaphylactic contraction of the isolated uterus can be obtained, when any one of a variety of human urinary gonadotrophic extracts is used as test antigen.

Precipitation experiments have shown that Pregnyl contains more than one antigen, and the presence of precipitins for human serum proteins, as well as for urinary antigens, could be quantitatively demonstrated in anti-Pregnyl sera. The antisera used in the precipitation tests were obtained from rabbits after periods of immunization of at least 3 months.

The presence of precipitins for human serum proteins may therefore be the result, either of loss of specificity occurring after prolonged immunization with a single antigen other than a serum protein, or of the actual presence of traces of serum proteins in the hormone extract used for immunization. The present experiments confirm the complex antigenic nature of Pregnyl, and show further that, of the antigens present, one is a species specific serum protein, while another is specific for urine. They confirm, therefore, also the demonstration of species specificity of urine previously shown by Rhein [1913], Kamekura [1925], Uhlenhuth and Seiffert [1929], and others.

When guinea-pigs were passively sensitized to one of the urinary extracts by means of serum from a rabbit which had received a long course of immunizing injections of that extract, the plain muscle showed a slight sensitivity to human serum proteins, but this was completely discharged when the muscle had responded to an effective dose of the urinary extract. On the other hand, active sensitization of a guinea-pig with the same extract produced a condition in which the plain muscle was proportionally more sensitive to human serum, in that it still gave a full reaction to this when it had reacted to the urinary extract used in sensitization and had thereby been rendered completely insensitive to a further similar application of the same extract. In seeking for an explanation of this anomaly, two points should be borne in mind. Active sensitization required only a very small dose of an efficient antigen [see Wells, 1925; Rosenau and Anderson, 1906], and there is good reason for expecting that serum proteins would act more efficiently and more quickly as antigens in this connexion than the antigenic substances peculiar to a urinary extract. Even though the proportion of serum protein present in a urinary extract is probably extremely small, it is accordingly not surprising to find the plain muscle of the actively sensitized guinea-pig showing relatively high sensitiveness to human serum proteins, which the minute quantity of these present in the dose of the urinary extract first applied is not capable of discharging, so that the plain muscle will subsequently react to a dose of human serum representing a large multiple of that to which it has already been exposed. On the other hand, serum from a rabbit subjected to a long and intensive course of immunization with the urinary extract may be expected to contain precipitating antibodies directed chiefly against antigens peculiar to urine, and in which antibodies for serum proteins form only a very small proportion. In the passive sensitiveness produced by such a serum anaphylaxis to human serum proteins will be a relatively very small component, and it is not surprising to find that it can be completely discharged by a reaction of the plain muscle to an adequate dose of the urinary extract.

It has previously been shown that the precipitins for serum proteins present in the anti-Pregnyl sera possess no antigonadotrophic properties. In testing for the presence of antibodies specific for gonadotrophic hormone from any source it has been important, therefore, to avoid the confusion which might arise from the presence in all extracts containing this hormone, of antigens having no hormonal significance, but carrying species specificity, such as serum proteins. It was found that in a uterus, actively or passively sensitized to urinary gonadotrophin, which had, for this reason, been desensitized to the specific effect of human serum, before testing its anaphylactic response to different gonadotrophic extracts, the sensitivity which it retained was for gonadotrophic extracts of urinary origin only. Gonadotrophic extracts obtained from human serum, or from human anterior pituitary lobes, possess no antigenic relationship to urinary gonadotrophins, other than the possession of a common serum protein constituent.

Even the fact that most gonadotrophins of urinary origin appear in these experiments to be antigenically similar only proves that they possess at least one urinary antigen in common, and gives no information as to the antigenic property of the gonadotrophic constituent. That antibodies are evoked by an antigen not necessarily related to the hormone is shown by the similarity of the results obtained with two extracts differing widely in gonadotrophic power, viz. Pregnyl and the male urine extract, M.U.G.; the former possessing 250 times the activity of the latter. It must be concluded, therefore, that this particular method, like the precipitation reaction, has failed to demonstrate the presence of hormone specific antibodies in antigonadotrophic sera. If, as is still possible, the hormone does possess antigenic powers, either alone or in combination with a nonspecific carrier antigen, its power of eliciting antibodies demonstrable by the anaphylactic as by the precipitin reaction is extremely weak, in comparison with that of the multiplicity of other antigens present in urinary gonadotrophic extracts.

Summary

Experiments in which antibodies were demonstrated by anaphylactic sensitization confirm those of precipitin tests on antigonadotrophic serum. The isolated uteri of sensitized guinea-pigs react specifically to contact with urinary extracts irrespective of their hormone content.

Sensitivity to human serum is also induced, but anaphylactic reaction to urinary extract is present even after specific desensitization to serum.

By active sensitization to urinary extracts, a higher degree of sensitivity to species (serum) antigen is induced than by passive sensitization. This

can be explained by the occurrence of traces of serum proteins in the urinary extracts.

The demonstrable antibodies evoked by urinary gonadotrophin are not hormone specific.

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THE EFFECT OF ANDROGENS ON THE MAMMARY GLAND OF THE FEMALE RHESUS MONKEY

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IT is now well known that many androgenic substances possess certain gynaecogenic properties and it is therefore not surprising that administration of androgens has been found to promote mammary development in the rat [Selye, McEuen, and Collip, 1936; Nelson and Gallagher, 1936; Astwood, Geschickter, and Rausch, 1937; Reece and Mixner, 1939], mouse [van Heuverswyn, Folley, and Gardner, 1939], and guinea-pig [Bottomley and Folley, 1938*a*]. These observations assume added significance in view of recent reports on the clinical use of testosterone propionate for the treatment of certain mastopathic conditions in women [see Turpault, 1937; Desmarest and Capitain, 1937; Loeser, 1938]. The rationale of such treatment must presumably lie in an attempt to reduce the oestrogenic stimulation of the breast by suppression of the gonadotrophic potency of the pituitary. In view of the fact that the agent used for this purpose is known to exert direct growth, and in some cases secretory, effects on the mammary gland in rodents, studies of the effects on the mammae of androgenic stimulation in primates are of considerable importance in assessing the safety or otherwise of the clinical use of male hormone in women.

Unfortunately, such studies on primates are very scanty, but since Geschickter and Astwood [1937] reported mammary alveolar growth following testosterone propionate administration in the rhesus monkey, while Folley, Guthkelch, and Zuckerman [1939] described papillomatous changes in the mammary duct epithelium of the same species after testosterone propionate administration and also observed alveoli to be present in some male glands after treatment, the importance of acquiring further data on the results of androgenic stimulation of the primate mammary gland needs little emphasis.

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METHODS

Material and experimental animals.

The following crystalline androgens were used: testosterone propionate, *cis*-androsterone, Δ^5-*trans*dehydro*iso*androsterone, and Δ^5-*trans*androstenediol. The experimental animals were pre-adolescent female rhesus monkeys. The weights of the animals at the time of the experiment ranged from 1,800 to 2,500 g., and in most cases the mammary gland was beginning its peripheral extension and elaboration. Ovariectomy was done under intraperitoneal 'Nembutal' anaesthesia, the right mammary gland being removed at the same time as a control. Injections were begun a few days after the operations. At the end of the injection period each animal was killed and the remaining mammary gland removed. In all cases save one the androgens were injected in solution in olive oil (the solvent in the case of Mm 413 was peanut oil), the oil volume being kept as low as possible. Data relating to the experimental animals and their treatment are given in Table I.

Histological methods.

Each gland, whether removed at operation or autopsy, was fixed and stained as a whole. At autopsy, however, the gland could be removed with the skin in order to lessen the distortion. Fixation in Bouin's fluid was followed by staining with alum carmine and clearing in cedarwood oil. Small blocks of tissue for microscopical study were cut from the whole mounts, sectioned, and restained with haematoxylin.

RESULTS AND DISCUSSION

Our material includes preparations from a series of animals receiving widely different total doses of testosterone propionate. Further, the control glands made it possible to arrange the animals in a series so that the effect of testosterone propionate was investigated not only on glands consisting of a few unbranched ducts, but also on a gland with alveoli and beginning lobule formation and glands at intermediate stages. In no case was there any evidence whatever that testosterone propionate had caused any growth of the mammary ducts (see Plate I, Figs. 1 and 2). The absence of connective tissue proliferation, and the fact that the terminal buds were thin-walled and ballooned, appeared to rule out growth of the gland by extension, while no increase in complexity of the duct systems could be detected. Furthermore, in none of our three animals with rudimentary glands (i.e. glands in which the duct system was not very extensive and in which there were no alveoli prior to treatment) was there any evidence that the treatment had caused alveolar develop-

ment. The control gland of Mm 490 (Plate I, Fig. 2) showed signs of beginning alveolar formation, and after treatment with testosterone propionate there was some proliferation and organization of alveolar elements. In the case of Mm 413 in which, at the beginning of the experiment, the mammae contained a fair amount of alveolar tissue, the gland after treatment was considerably more dense than the control (Plate I, Fig. 3). Examination of the whole mount and of histological sections (Plate I, Figs. 4 and 5) indicates that the change is largely due to distension of the ducts and alveoli by accumulation of secretion, but there has also been

Table I

No. of monkey	Body-weight at operation g.	Androgen administered	Total amount mg.	Injection period days	Remarks
Mm 493	2250	Testosterone propionate	100	68	10 mg. once a week.
Mm 490	1945	,, ,,	100	10	Four spaced injections of 25 mg.
Mm 413	2470	,, ,,	388	65	Weekly injections of 40 mg. were given over the greater part of the injection period.
Mm 488	1960	,, ,,	1000	65	Injections of 100 mg. given twice weekly beginning 5 days after operation.
Mm 487	2435	,, ,,	2000	65	Injections of 200 mg. given twice weekly beginning 5 days after operation.
Mm 489	2000	*cis*androsterone	1350	60	Five equal injections of 30 mg. per week.
Mm 491	1855	Δ^5-*trans*dehydroiso-androsterone	1500	60	Three spaced injections of 60 mg. per week.
Mm 492	2080	Δ^5-*trans*androstenediol	450	60	Five injections of 10 mg. per week.

some growth of new alveoli. Under the high power (Plate I, Fig. 5) can be seen regions in which the glandular epithelium is puckered in an irregular manner, reminiscent of 'heaping' of the duct epithelium described by Folley *et al.* [1939]. Treatment with testosterone propionate, therefore, in the monkey, appears only to cause alveolar growth in glands already containing alveolar tissue. Since details of Geschickter and Astwood's [1937] experiments are lacking and control glands are not shown, it is not possible satisfactorily to discuss their results in relation to ours, beyond pointing out the possibility, in view of our findings, that alveolar tissue existed in the glands of their animals prior to injection.

Our experiments, further, clearly show that despite the fact that except in the presence of pre-existing alveoli, testosterone propionate caused no mammary growth, it nevertheless markedly affected the mammary duct system by causing gross dilation of the ducts, due to distension by secretion from the epithelium. This result was obtained in all experiments with testosterone propionate (see Plate I, Figs. 1, 2, and 3, which are typical). In all animals receiving testosterone propionate a colourless, serous fluid could be expressed from the nipples. In the case of Mm 413 the secretory

process had proceeded to the point at which the ducts were characterized by a cystic condition, the bulbous enlargements at their ends being particularly noticeable (Plate I, Fig. 3). Under the conditions of our experiments, therefore, treatment with total doses of testosterone propionate ranging from 100 mg. to 2,000 mg. causes secretory changes in the mammary epithelium of the rhesus monkey.

As regards other androgenic substances, treatment with Δ^5-*trans*-dehydro*iso*androsterone affected a rudimentary gland in a way similar to testosterone propionate, though the degree of duct dilation was not as great, while in the one experiment with Δ^5-*trans*androstenediol only a small degree of dilation was apparent. In this case, however, it was only possible to administer a comparatively low dose of the androgen because of its low oil solubility, together with the advisability of keeping the injected oil volume within reasonably low limits. Nevertheless, though the dosage of Δ^5-*trans*androstenediol was somewhat greater than that of testosterone propionate given to Mm 413, the effect on the mammary gland in the latter case was far greater than in the former. Lastly, it is important to note that administration of a high dose of *cis*androsterone had practically no noticeable effect of any sort on a mammary gland. In this connexion it is of interest that *cis*androsterone was the least active of a series of androgens in causing mammary duct growth in the male mouse [van Heuverswyn *et al.*, 1939]. It should be stated that the glands taken from the animals receiving the last three androgens contained no alveolar tissue before treatment and none after.

Taken as a whole, these results indicate the existence of species differences as regards the effect of androgens on the mammary gland. Treatment with androgens appears to cause no mammary duct growth in the spayed pre-adolescent female rhesus, neither does such treatment promote alveolar development except in glands in which alveoli already exist. On the other hand, in the mouse [van Heuverswyn *et al.*, 1939] androgenic stimulation causes duct development in the male. In the rhesus monkey the most consistent and striking effect of androgens (particularly with testosterone propionate and hardly noticeable with *cis*androsterone) on the mammary gland consists of the induction of a secretory condition of the epithelial cells, which in turn leads to dilation of the ducts. Further, in glands containing alveolar tissue, testosterone propionate appears to cause an abnormal folding or 'heaping' of the glandular epithelium. In a moderately mature gland (Mm 413) these processes have resulted in an abnormal condition.

In the absence of parallel experiments on hypophysectomized monkeys it is impossible to say whether or not the secretory effect is mediated by the pituitary, though Reece and Mixner [1939], who observed a similar

secretory phenomenon in the mammary gland of the rat after treatment with testosterone propionate [see also Selye *et al.*, 1936], concluded that the pituitary was involved, since an increase in the 'lactogen' content (assayed on pigeons) of the latter occurred.

In view of the possibility that, in the human, testosterone propionate might induce abnormal changes in the mammary epithelium similar to those found in the rhesus monkey, the need for caution in the use of testosterone propionate for clinical purposes in women is obvious. On the other hand, in considering the applicability of these results to women it must be borne in mind that the total doses per kg. given in these experiments are much higher than the doses usually administered to women. Nevertheless, the comparative freedom of *cis*androsterone from the undesirable property under discussion coupled with the fact that androsterone is capable of causing pituitary 'suppression' in the guinea-pig [Bottomley and Folley, 1938*b*] suggests that its possible application for the treatment of 'mastitis' in women would be worthy of clinical trial.

SUMMARY

1. Treatment of pre-adolescent ovariectomized female rhesus monkeys with testosterone propionate induced a secretory condition in the mammary gland epithelium leading to dilation of the mammary ducts.

2. Testosterone propionate even in high doses did not cause extension or arborization of the mammary ducts nor did it promote alveolar development except in glands in which alveoli already existed. In a moderately mature gland the treatment had induced, in addition to development and organization of the alveoli, irregular 'heaping' of the glandular epithelium.

3. Of three other androgens given in high doses, each to a single animal, Δ^5-*trans*dehydro*iso*androsterone and Δ^5-*trans*androstenediol caused some secretion and duct dilation in glands containing no alveoli while *cis*-androsterone gave only a very slight reaction.

4. In view of these results it is suggested that *cis*androsterone might be preferable to testosterone propionate for the treatment of 'mastitis' in women.

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(a)   FIG. 1.   (b)
(a) Control mammary gland from Mm 487. × 2.
(b) Mammary gland of Mm 487 after injection of 2000 mg. testosterone propionate over 65 days, showing gross dilation of the ducts. Note the absence of duct growth and alveolar development. × 2.

(a)   FIG. 2.   (b)
(a) Control mammary gland from Mm 490. × 2.
(b) Mammary gland of Mm 490 after injection of 100 mg. testosterone propionate over 10 days. The duct system is dilated and there has been alveolar growth, but no duct extension has occurred. × 2.
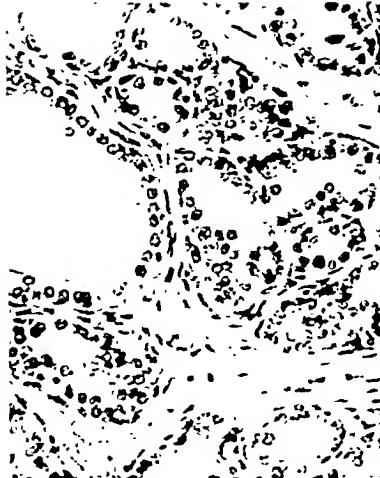
FIG. 5. High power photomicrograph of a portion of the alveolar tissue in the mammary gland of Mm 413 after treatment with testosterone propionate, showing irregular puckering of the glandular epithelium. × 165.

(a)   FIG. 3.   (b)
(a) Control mammary gland from Mm 413 showing extensive duct system and alveolar development. × 2.
(b) Mammary gland from Mm 413 after injection of 388 mg. testosterone propionate over 65 days. Note the gross dilation of the ducts and their thin-walled, balloon-shaped endings at the periphery. Note also the dense alveolar tissue which now extends over the whole gland. × 2.
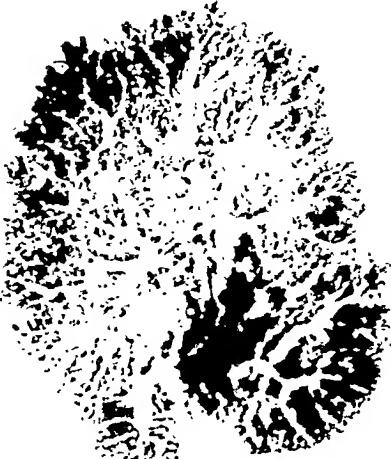
(a)   FIG. 4.   (b)
(a) Photomicrograph of portion of control mammary gland from Mm 413. The ducts are small and alveolar tissue sparse. × 6.
(b) Photomicrograph of portion of mammary gland from Mm 413 after treatment with testosterone propionate. The ducts are large and filled with secretion and there are numerous lobules of alveoli. × 6.

THE RESPONSE OF INBRED MICE
TO OESTRONE

By C. W. EMMENS

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THE Allen-Doisy vaginal cornification test is used almost exclusively for the biological assay of oestrogenic preparations. In its commonest form, from one to six injections of the test material are given to spayed rats or mice during the course of two or three days, and vaginal smears are taken on the third, fourth, and perhaps the sixth day. These smears are assessed according to their cellular composition, the simplest method of scoring being to record those with cornified or nucleated epithelial cells but no leucocytes as positive, and the rest as negative. Other methods are in frequent use, in which the various stages of the oestrous cycle are recorded and given key letters, or in which each smear is given a numerical value according to the degree of cornification, &c., observed. The main drawback to most of these more involved ways of assessing the smears is that the results cannot be dealt with by the fairly simple mathematical technique applicable to quantal (all or none) responses, as they cannot usually be expressed by linear regression functions. It is, moreover, doubtful whether any increase in the accuracy of estimation is obtainable by such methods.

In this Institute, therefore, it has been the practice to treat the response of the spayed mice which are used in such assays as strictly quantal, and to employ y/log-dose co-ordinates in the calculation of the regression lines, where y is the normal equivalent deviation, or probit [cf. Gaddum, 1933; Bliss, 1935; Irwin, 1937]. It has been found that such lines fit the recorded data, and justify the assumption that the logarithms of the individual effective doses are normally distributed. The slope of a fitted line is inversely proportional to the standard deviation of the logarithms of the individual effective doses. Steep lines are, therefore, characteristic of a low degree of variation in response, and the lower the numerical value of the slope, the greater the variation encountered in the tests.

It has also been shown [Emmens, 1939] that no significant deviations occurred in the slope of the dose/response line for spayed albino mice injected with international standard oestrone over a period of 7 months, during which 14 determinations of the slope were made at fortnightly intervals. Subsequent work has confirmed this result, and it is therefore valid to compare the slopes of dose/response lines for different colonies, although the data for these lines may have been gathered at different times.

MATERIAL AND METHODS

A full account of the technique of tests of this type has already been given [Emmens, 1939]. Briefly, injections of oestrone in arachis oil are made into groups of about 20 mice, at several dose levels, one injection being given on each of two consecutive days. Smears are taken on the third day, either at 4 p.m. or at 11.30 p.m. (see below) and at 10 a.m. and 4 p.m. of the fourth day. The volume of oil in which each injection was made was kept uniform at 0·1 ml. for most of the tests, but in those dealing with the inbred strain, and in a further control test using albino mice, the volume varied between 0·05 and 0·2 ml. in different groups. As will be seen below, there is no reason to believe that alteration of the injection volume within these limits has an effect on response.

The albino mice, to which reference has already been made, are taken from the Institute's farm stock, which has been inbred to a very slight degree, in that it is descended from three original pairs of mice, and has not received additions since its inception, but mating has otherwise been at random.

The inbred mice are the descendants of derivatives of Strong's pure line C.B.A. (F_{27}), substrain Grüneberg (F_{38}, 1938), kindly provided by Dr. H. Grüneberg. The animals used in the tests were of generations F_{40} and F_{41}, of this line, brother-sister mating being abandoned in the F_{39} generation as far as the mice intended for ovariectomy were concerned. These mice should therefore have been as genetically homozygous as one could hope to obtain. They were ovariectomized by the usual technique over a period of three or four months, and primed two weeks before use in the tests with 0·5 μg. of international standard oestrone, which was used throughout all tests.

Regression lines were calculated by fitting a provisional line by eye, and the weighting coefficients to be used in the calculation of the final line were determined from it. Responses of 0% or 100% were included by the method of Fisher [1935]. The standard error of the determination of the slope is then given by

$$\sigma_b = \frac{1}{\sqrt{\{S[nw(x-\bar{x})^2]\}}},$$

where n = the number of animals, w = the weight factor, and x = the logarithm of the dose, for each group.

RESULTS

Dose/response lines for albino mice.

The slope of the dose/response line for albino mice, when smears were taken at 11.30 p.m. on the third day of the test and at 10 a.m. and 4 p.m.

on the fourth day, and when the volume per injection was kept at 0·1 ml., has already been discussed [Emmens, 1939]. The series of control determinations made at that time (10/5/37–6/12/37) on totals of between 40 and 140 mice per test showed the slope to be 5·485±0·517, the standard error being estimated from the observed distribution of values of the slope. A smaller value for the standard deviation of the slope is obtained

if the formula: $\dfrac{1}{\sigma_b^2} = S\left(\dfrac{1}{\sigma_{b'}^2}\right)$ is employed, where σ_b = the standard error

of the average value of the slope found, and $\sigma_{b'}$ is the standard error of any individual determination. This value, 0·349, could justifiably be employed in the calculations that follow, but, as it happens, it does not affect our conclusions whichever value we use.

A second colony of albino mice from the same stock was used in a further determination of the slope on 7/3/38, under the same test conditions. On this occasion 120 mice were injected and gave a slope of 5·453±0·825 for the regression line. Eighty mice of a third and similar colony were later employed on 8/8/39 for another determination, with slight alterations in technique. Smears were taken at 4 p.m. on the third day of the test, instead of at 11.30 p.m., and the volume of oil per injection varied between 0·05 and 0·2 ml. This enabled us to use a constant concentration of oestrone in most groups of mice. The slope for this colony was 4·992±0·884, and was clearly not significantly affected by the change in technique (Table I).

Dose/response lines for the inbred mice.

The slope of the line for the inbred mice was determined four times, with the technique as outlined for the third colony of albinos. Table I gives the necessary data relating to these determinations.

The average value of the slope is 3·375±0·397, using the formula $\dfrac{1}{\sigma_b^2} = S\left(\dfrac{1}{\sigma_{b'}^2}\right)$ as before, since the number of determinations is too small for

a direct estimate of the standard error. This value differs significantly from that for the first albino colony (*P* lies between 0·002 and 0·0001, according to which value of the standard error is used for the albinos), that for the 2nd albino colony (*P* = 0·025 approx.), but not from that for the 3rd colony (*P* < 0·05). However, there is little justification for considering the slopes of the lines for the three albino colonies separately, as they do not differ significantly from one another, and, when the results for the albinos are considered together, the average slope for the inbred mice is found to be highly significantly less than the average for the albinos (*P* < 0·001).

DISCUSSION

The fact that the slope of the dose/response lines for the responses of the Strong C.B.A. mice to oestrone is less than that for the responses of randomly mated albino mice, means that the inbred mice are actually more variable in response than are the albinos. It seems highly improbable that the inbred strain can be heterozygous for genes which could be responsible for the variability, so that we are led to the rather surprising conclusion that the factor causing variation in the response of mice to oestrone is unlikely to be dependent on differences in their genetic constitution.

Table I. *Data relating to the slope of the dose/response lines for spayed mice injected with oestrone*

Date of test	Nature of animals	Total number of mice in test	Slope of line	Standard error of slope
10/5/37 to 6/12/37 (14 tests).	*Albino mice* 1st colony	660	5·458	0·517 (0·349 *see text*)
7/3/38	2nd colony	120	5·453	0·825
8/8/39	3rd colony	80	4·992	0·884
—	Weighted mean for albino mice	860	5·353	0·418
30/5/39	*Inbred C.B.A. mice*	40	3·934	1·430
12/6/39	,, ,,	40	1·259	1·228
26/6/39	,, ,,	80	3·486	0·634
24/7/39	,, ,,	80	3·774	0·604
—	Weighted mean for inbred mice	240	3·375	0·397

While the lowest dose of oestrone to which an individual albino mouse will react varies from time to time, the logarithm of this dose tends to bear a constant relation to the logarithm of the dose causing 50% of positive responses in the colony as a whole [Emmens, 1939]. There are, therefore, innate differences in the sensitivity of these mice, which at the time of investigation were thought to be most probably due to genetic causes. This view cannot reasonably be held now, as whatever differences exist between the individuals of the C.B.A. stock must presumably be environmental in origin. The previous study also showed that the response of an albino mouse does not depend to any detectable extent on its age, weight, or its previous fortnightly use in tests, as long as it has received sufficient oestrogen within a short period of its use in any particular test [cf. also Marrian and Parkes, 1929]. As this is probably true for the C.B.A. mice, one is left to conclude that in this particular pure line, if not in others, uniformity of nurture is more important than uniformity of inheritance in determining the response to oestrogenic stimulation.

SUMMARY

In a pure line of mice, a derivation of Strong's C.B.A. strain, the responses to oestrone were found to be more variable than those of randomly mated albinos

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MUTUAL ANTAGONISM BETWEEN OESTROGENS AND ANDROGENS

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IN the early stages of work with oestrogenic and androgenic extracts, much consideration was given to the question of antagonistic or synergistic action between the two. Juhn, D'Amour, and Womack [1930] found no interaction between them when given simultaneously· in the form of extracts to capons, but Schoeller and Gehrke [1933] later reported a synergistic action under these conditions. However, workers using crystalline substances have been unable to confirm these results. Callow and Parkes [1935], Morató-Manaro, Albrieux, and Buño [1935], and Gley and Delor [1937] agree in finding that the response of the capon comb to various androgens is decreased by the simultaneous administration of oestrogens. Mühlbock [1938] has shown that a daily dose of 5 mg. of oestradiol benzoate will completely abolish the response of the capon comb to 100 μg. daily of androsterone, and in a recent paper Emmens [1939a] produced curves showing the progressive decrease in the response of groups of capons receiving a single injection of 150 μg. of testosterone propionate together with increasing quantities of oestradiol benzoate injected at a different site. Although as little as 10 μg. caused partial inhibition of the response, as much as 10 mg. did not completely abolish it.

In male mammals the interaction of the two groups *of substances* may be classed either as synergistic, since various workers have shown that a co-operative effect may be observed on the secondary sexual organs, *or as* antagonistic, since an adequate dosage with androgens will protect the male from the effects of oestrogens. In female mammals it has been demonstrated by Robson [1936] and Hain [1937] that testosterone and testosterone propionate in large doses will inhibit the response of spayed rats and mice to oestrone; while Deanesly and Parkes [1937] found that *trans*androstenediol, an androgen which causes vaginal cornification in spayed mice, may itself be inhibited by testosterone propionate. Further data on such inhibition, using androgenic extracts from various sources, were given by Emmens and Parkes [1938].

It is apparent, therefore, that whatever synergistic action may exist in some species when relatively small quantities of an oestrogen are given

together with an androgen, there is an antagonism between the action of these substances when relatively large doses of an oestrogen are given simultaneously with an androgen in the capon comb-growth test, and also when large doses of testosterone are given together with an oestrogen in the Allen-Doisy vaginal cornification test in rats or mice. In the present paper further consideration is given to these phenomena, using various oestrogens, given by different routes, as antagonists to androsterone, and various androgens as antagonists to oestrone, in the capon comb-growth and Allen-Doisy tests respectively.

Material and methods

Tests on the induction of growth in the Brown Leghorn capon's comb were carried out as described previously [Emmens, 1939b] using a 3-day test period. Androsterone in 0·1 ml. arachis oil was given by injection or by inunction on to the comb, once daily during the test period. Injections or inunctions of the oestrogen, also in oil, were given simultaneously, and into the opposite breast muscles when both substances were administered by injection. In some cases the volume of oil carrying the oestrogen had to be increased above 0·1 ml. Comb growth is expressed as the increase in length plus increase in height, an average value being taken from a group of 5 birds in all cases.

Allen-Doisy tests were made with spayed mice, the general technique also being as described by Emmens [1939b], one injection of each substance being given on each of two consecutive days. Groups of 5 mice were used, the oestrogen being injected subcutaneously on one side of the animal, and the androgen on the other side. The volume of oil carrying the oestrogen was 0·1 ml. per injection, that carrying the androgen varied in different tests from 0·05 to 0·2 ml. During the period covered by these latter tests, which must be considered as of a preliminary nature, control groups of 20 mice receiving a total of 0·1 μg. of oestrone gave between 60% and 80% of positive responses. The results are therefore grouped together without consideration of the date of any particular assay, since the sensitivity of the mice may be considered to have remained approximately constant.

Oestrogens as anti-androgens

Oestrone. Table I shows the comb growth resulting from the simultaneous administration of androsterone and oestrone to capons by various combinations of routes. The inhibitory effect of different doses of inuncted oestrone on the response to a constant total dose of 600 μg. of injected androsterone gives an approximately linear relationship over the greater part of the curve when the comb growth resulting from the simultaneous administration of the two, expressed as a percentage of the growth in a

control group receiving no oestrone, is plotted against the logarithm of the total dose of oestrone (Fig. 1). It is, moreover, possible, by this method of applying oestrone, to inhibit the response completely. A similar type of relationship appears to hold for injected oestrone, but complete inhibition of the response has not been demonstrated under these conditions.

Table I. *The inhibitory effect of oestrone on the response of groups of 5 capons to injected androsterone*

Date of test	Total dose of androsterone μg.	Total dose of oestrone μg.	Route of administration of oestrone	Response in mm.	Response as % of that of control groups
20/4/39	300	—	—	3·2	100
,,	300	250	inuncted	0·0	0
,,	300	500	,,	0·7	22
,,	300	1000	,,	−0·8	−25
4/5/39	600	—	—	4·7	100
,,	600	3	inuncted	4·7	100
,,	600	6	,,	3·6	77
,	600	12	,,	2·6	55
,	600	24	,,	1·0	21
,,	600	48	,,	1·4	30
,,	600	150	,,	−0·1	−2
,,	600	600	injected	4·1	87
,,	600	1500	,,	4·3	92
,,	600	3000	,,	2·9	62
25/5/39	600	—	—	5·1	100
,,	600	6000	injected	2·8	55
,,	600	12,000	,,	2·2	43
22/6/39	3 (inuncted)	—	—	4·1	100
,,	,,	45	inuncted	1·9	46

By expressing the comb growth of any group as a percentage of that shown by the controls it is possible to compensate for the time-to-time variation in response shown by the latter, although, strictly speaking, it has yet to be shown that the nature of the dose/response curve for oestrone as an anti-androgen is not affected by alteration in the sensitivity of the birds.

A comparison of the amount of inuncted oestrone effective in causing a 50% inhibition of the response with that needed to give the same degree of inhibition by injection, shows that about 16 μg. by inunction are equivalent to 7,500 μg. by injection, a ratio of 1 to 470. The increase of efficiency on inunction is therefore greater than that found for androsterone, when its action in causing comb growth is considered, since about 170 times the inuncted dose of androsterone is needed to cause an equivalent amount of comb growth by injection [Emmens, 1938]. Tested against inuncted androsterone (Table I) by applying a mixture of the two, oestrone gave the degree of inhibition expected from its action against

injected androsterone, showing that the relative effectiveness of both compounds is maintained when both are given by inunction.

Oestradiol. Table II shows the data for oestradiol given by inunction or injection together with injected androsterone. The slope of the % response/log.-dose curve for dosage by inunction (Fig. 2) is less than that
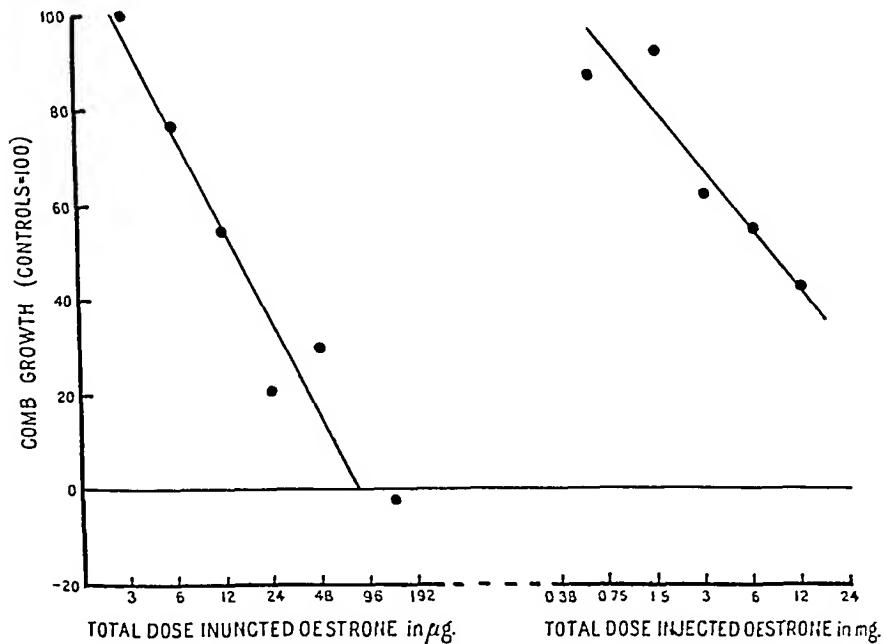
Fig. 1. Inhibition of the growth of the combs of Brown Leghorn capons receiving a constant total dose of 600 µg. of androsterone by injection, together with various amounts of injected or inuncted oestrone. 5 birds per group.

for oestrone, and this difference, therefore, precludes any accurate comparison of the potencies of the two. In low doses oestradiol appears to be more effective per unit weight than oestrone, but less effective in higher doses. This change of relative effectiveness seems most probably to be related to the rate of absorption of the compounds; high doses of oestradiol are perhaps more quickly removed from the site of action than corresponding doses of oestrone. By injection, on the other hand, oestradiol is relatively more effective than oestrone at all dose levels. This again is explicable on the assumption that oestradiol passes more rapidly into the circulation than does oestrone, even though it may be as rapidly eliminated, since the substances must reach the comb in effective amounts for inhibition to be observed, and the slower absorption of oestrone from the site of injection may be a factor in causing its relatively low efficiency by injection when compared with oestradiol.

Table II. *The inhibitory effect of oestradiol on the response of groups of 5 capons to injected androsterone*

Date of test	Total dose of androsterone μg.	Total dose of oestradiol μg.	Route of administration of oestradiol	Response in mm.	Response as % of that of control groups
4/5/39	600	—	—	4·7	100
,,	600	300	injected	4·1	87
,,	600	1500	,,	3·9	83
22/6/39	600	—	—	4·4	100
,,	600	1·5	inuncted	4·2	96
,,	600	3	,,	3·6	82
,,	600	6	,,	3·4	77
,,	600	12	,,	3·2	73
,,	600	24	,,	3·4	77
,,	600	48	,,	2·5	57
,,	600	3000	injected	1·0	23
,,	600	6000	,,	0·7	16
,,	600	12,000	,,	−0·8	−18

Diethylstilboestrol. The data for the synthetic oestrogen, diethylstilboestrol, are shown in Table III. It has been reported by Mühlbock [1939] that this compound, when given by inunction, is ineffective in inhibiting the action of androsterone on the capon's comb. However, as shown
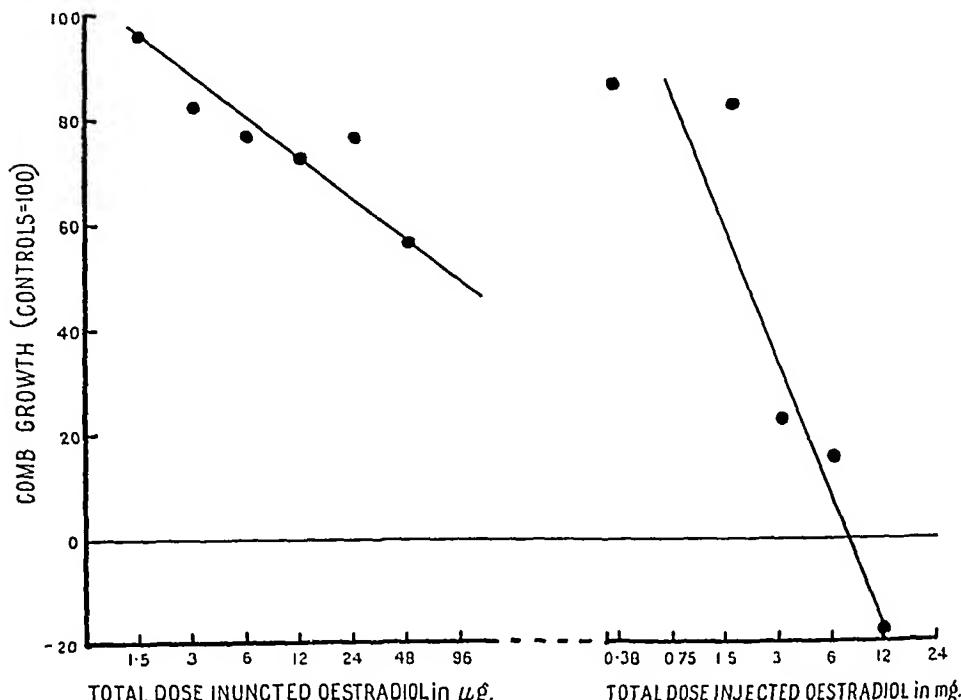
FIG. 2. Inhibition of the growth of the combs of Brown Leghorn capons receiving a constant total dose of 600 μg. of androsterone by injection, together with various amounts of injected or inuncted oestradiol. 5 birds per group.

in Table III, complete inhibition was observed when 300 μg. of andro-
sterone were injected, and 3 mg. of diethylstilboestrol were inuncted
on to the comb. Further tests showed that the anti-androgenic activity
of diethylstilboestrol, when given by inunction, is very low in comparison
with that of oestrone and oestradiol, although its oestrogenic potency is
intermediate when tested on spayed mice. A 50% inhibition is caused by
about 1·6 mg., as shown by the tests listed in Table III (see also Fig. 3),
giving a potency of about 1% that of oestrone under the same conditions.

Table III. *The inhibitory effect of diethylstilboestrol on the response of groups*
of 5 capons to injected androsterone

Date of test	Total dose of androsterone μg.	Total dose of diethyl-stilboestrol μg.	Route of administra-tion of diethyl-stilboestrol	Response in mm.	Response as % of that of control groups
20/4/39	300	—	—	3·2	100
,,	300	3000	inuncted	−0·5	−16
4/5/39	600	—	—	4·7	100
,,	600	75	inuncted	4·5	96
,,	600	300	,,	3·0	64
,,	600	1200	,,	2·9	62
,,	600	3000	injected	2·5	53
,,	600	30,000	,,	0·3	6
25/5/39	600	—	—	5·1	100
,,	600	3000	inuncted	2·0	39

When injected, however, diethylstilboestrol has about twice the potency
of oestrone, and its efficiency by injection is not markedly different from
its efficiency by inunction, since 3 mg. of injected diethylstilboestrol caused
just under a 50% inhibition of comb growth.

This remarkable difference between the natural and synthetic oestrogens
may be explained by either of two contrary suppositions. Either diethyl-
stilboestrol is very rapidly absorbed from the surface of the comb and
distributed by the circulation, and the effect of inunction is therefore
similar to that of injection; or it is very inadequately absorbed through
the surface, and cannot therefore exert its full effect. In view of the high
solubility of diethylstilboestrol in body fluids, as shown by its rapid
absorption from tablets of the compressed material implanted beneath
the skin, the former explanation seems the more likely.

A comparison of the amounts of each oestrogen needed to produce a
50% inhibition of the action of 600 μg. of androsterone is shown in Table IV.

Androgens as anti-oestrogens

The androgens were injected into groups of 5 spayed mice per test,
simultaneously with a daily dose of 0·06 μg. of oestrone, giving a total
dose of 0·12 μg. of oestrone. The results are shown in Table V. From

previously established dose/response curves [cf. Emmens, 1939b], it is known that, since controls on 0·1 μg. gave 60–80% of positive responses, 0·12 μg. of oestrone would, alone, give 75–90% of positive responses.
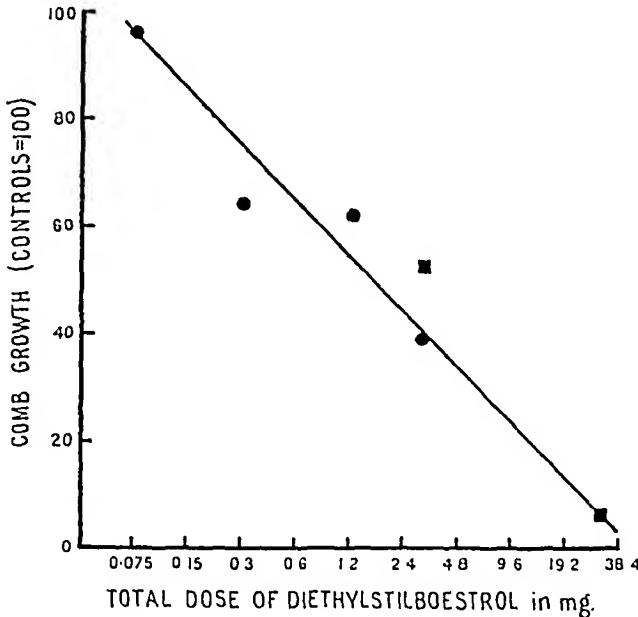
FIG. 3. Inhibition of the growth of the combs of Brown Leghorn capons receiving a constant total dose of 600 μg. of androsterone by injection, together with various amounts of diethylstilboestrol. 5 birds per group.
●—inuncted diethylstilboestrol
■—injected diethylstilboestrol

Taking the lower response as a margin of safety, we can calculate that, if in a group of 5 mice we expect 75% of positive responses, 1 or no mice will give positive responses in only 1·6% of cases, but that 2 or less will react positively in as many as 10·4% of cases. With 5 mice per group,

Table IV. *Approximate amounts in μg. of different oestrogens which, when given by inunction or by injection, cause a 50% inhibition of the response of the capon comb to 600 μg. of injected androsterone*

Substance	By inunction	By injection	$\left[\dfrac{\text{Dose by injection}}{\text{Dose by inunction}}\right]$
Oestrone	16	7500	470
Oestradiol	96	1750	180
Diethylstilboestrol	1600	3000	2

therefore, if none or only one mouse reacts positively, we may accept the test as showing a significant depression of the oestrogenic activity of 0·12 μg. of oestrone, but if 2 or more react positively there may be no real depression of activity. This criterion is sufficient for our purpose,

which is to compare approximately the anti-oestrogenic potencies of the androgens used.

Testosterone showed significant inhibition of oestrone in total doses of 1·0 and 0·5 mg., but not in a dose of 0·25 or 0·125 mg. The lowest effective dose of this compound may therefore be accepted as about 0·5 mg. The lowest effective doses of the other androgens, listed in Table V, were calculated by a similar method. Testosterone, methyl testosterone, andro-

Table V. *The inhibitory effect of different androgens on the response of groups of 5 ovariectomized mice to 0·12 μg. of oestrone*

Substance	Total dose mg.	Number of mice +ve	Lowest effective dose mg.
Testosterone	1·0	0	
	0·5	0	0·5
	0·25	3	
	0·125	3	
Methyl testosterone	2·0	1	
	1·0	1	0·5
	0·5	1	
	0·25	3	
Androstenedione	2·0	0	
	1·0	0	
	0·5	1	0·5
	0·25	3	
	0·125	4	
Androstanediol	2·0	0	
	1·0	1	
	0·5	1	0·5
	0·25	2	
	0·125	4	
Androstanedione	2·0	5	—
	1·0	5	—
Androsterone	2·0	3	—
	1·0	2	—
	0·5	5	—
*trans*Dehydroandrosterone	2·0	3	—
	1·0	4	—

stenedione, and androstanediol are found to be approximately equal in potency as anti-oestrogens, while androsterone, *trans*dehydroandrosterone, and androstanedione showed no significant inhibitory effect in the dosage tested (up to 2·0 mg.). The androgenic activities of the four active anti-oestrogens are, of course, not equal, whether tested on capons or mammals. Testosterone has 6 or 7 times the potency of androstenedione and 4 or 5 times that of androstanediol, when tested on the seminal vesicles and prostate gland of castrated rats [Tschopp, 1936], while methyl testosterone is rather more active than testosterone [Deanesly and Parkes, 1936]. In cases where it is desirable to inhibit endogenous oestrogens in the human female, it would appear that the use of androstenedione or andro-

stanediol, preferably the former, might give as satisfactory a result as testosterone, but without producing the extensive masculinization seen when *the latter compound is employed.*

SUMMARY

1. Oestrone, oestradiol, and diethylstilboestrol inhibit the response of the capon's comb to androsterone. When given by inunction, oestrone and oestradiol do not differ markedly in effectiveness, but the latter gives a very flat dose/response curve. Diethylstilboestrol is relatively inefficient by this route. Oestradiol is the most potent anti-androgen by injection, followed by diethylstilboestrol and then oestrone. The ratio of the dose by injection to the dose by inunction required in each case to produce a 50% inhibition of the response to 600 μg. of injected androsterone is approximately 470:1 for oestrone, 180:1 for oestradiol, and 2:1 for diethylstilboestrol.

2. Testosterone, methyl testosterone, androstenedione, and androstanediol are about equally effective in inhibiting the response of spayed mice to oestrone, a total dose of 0·5 mg. of each producing significant inhibition. Androsterone, *trans*dehydroandrosterone, and androstanedione were ineffective in doses up to 2·0 mg.

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THE EFFECT OF VASOPRESSIN, SEX HORMONES AND ADRENAL CORTICAL HORMONE ON BODY-WATER IN AXOLOTLS

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THE changes which occur in the body-weight of frogs, axolotls, and aquatic reptiles as a result of the injection of vasopressin are generally ascribed to changes in body-water [e.g. Brunn, 1921; Bělehrádek and Huxley, 1927; Heller, 1930; Steggerda, 1931; Steggerda and Essex, 1934; Rey, 1935; Steggerda, 1937; Boyd and Brown, 1938; Boyd and Whyte, 1938; and Boyd and Dingwall, 1939]. The mechanism of the change appears to differ somewhat in different species, but in amphibia it appears to be due both to the increased absorption of water through the skin [Steggerda, 1931] and to antidiuresis [e.g. Rey, 1935; Pasqualini, 1938], with the consequent storage of excess water in muscle, subcutaneous, and other tissues [Steggerda and Essex, 1934; Boyd and Brown, 1938]. In view of the simplicity of the technique necessary to follow gross changes in body-water in these animals, it was of interest to inquire whether or not sex hormones and corticosterone have the same power to cause water retention in amphibia as they have in mammals [Krohn and Zuckerman, 1937; Thorn and Harrop, 1937; Guthkelch and Zuckerman, 1937; Thorn, Nelson, and Thorn, 1938; Zuckerman, 1939].

MATERIAL AND METHODS

Animals.

Seventeen axolotls, varying in weight between 25·60 and 116·62 g., were used. Each animal was kept in a separate glass tank, 14 in. tall, 12 in. long, and 8 in. wide, half-filled with tap-water. The water was changed twice weekly and at the same time the animals were fed with worms, or with chips of meat, until they were satisfied. No experimental observations were made on feeding-days for, apart from the question of the increase in body-weight due to the ingested meat, it was found that the axolotls often regurgitated their food when disturbed.

Method of weighing.

The animals were taken from their tanks by hand, and adhering water was mopped off with a smooth towel. They were then put into a previously dried beaker and weighed on a swing balance. Immediately after weighing

the animals were returned to their tanks, and the beaker was reweighed. The difference between the two weighings gave the weight of the animals.

Experimental error in weighing.

Six axolotls were weighed ten times in succession on two separate occasions. It was found that in only two of the 120 weighings did the weights vary by as much as 1%, the mean variation being $0.26 \pm 0.02\%$, the standard deviation of the distribution being $\pm 0.24\%$. In view of the magnitude of the changes dealt with in this study, this experimental error is negligible.

Injections.

The animals were firmly held on their backs in a towel, and injections were made intraperitoneally to the right or left of the midline with a 1 c.c. glass tuberculin syringe.

The following hormone preparations were used:

Vasopressin. Three dilutions of vasopressin, containing 1 unit, 2 units, and 3·3 units per c.c. respectively, were prepared from a stock preparation containing 20 units per c.c. by diluting with a fluid made up of 5·0 c.c. of absolute alcohol and 0·2 c.c. of 10% NaOH made up to 100 c.c. with 0·9% NaCl. This diluting fluid was chosen as it is a fairly effective 'aqueous' solvent for oestrone, and as one set of control observations carried out with this solvent would therefore cover both vasopressin and 'aqueous oestrone' experiments.

Oestrone. One set of experiments was carried out with an aqueous solution containing 25 μg. per c.c. The solvent used was the same as that for diluting the vasopressin. A further set was carried out with a solution of 1 mg. per c.c. of arachis oil.

Testosterone propionate. The only preparation used was a solution of 50 mg. per c.c. of arachis oil.

Progesterone. A solution of 10 mg. per c.c. of arachis oil was used.

Cortical hormone. Three preparations were used: (1) a solution of 5 mg. of desoxycorticosterone per c.c. of arachis oil; (2) an adrenal cortical extract of which each c.c., equivalent to 40 g. of fresh beef adrenal gland, was assayed to contain 4 (survival) rat-units ('Cortin', Upjohn Company); and (3) an adrenal cortical extract of which each c.c. was equivalent to 75 g. of adrenal cortex ('Eucortone', Allen & Hanbury). The solvent for the Upjohn 'Cortin' is 0·9% NaCl and 10% alcohol, and that for the Allen & Hanbury 'Eucortone', water.

All injections were given at approximately the same time of day with an interval of not less than three days between successive experiments. The animals were weighed immediately before being injected and again 30 minutes later. As a general practice, twelve further weighings were

made at hourly intervals from the time of injection, and a final weighing was made 24 hours after the start of the experiment.

Control observations.

Eighteen control observations were made on animals receiving no injections. The animals were weighed at the times stated above in order to obtain evidence about individual variation in weight over a period of 24 hours.

Every experiment was also controlled, usually at the time it was carried out, by injecting a control animal with a volume of solvent equal to that given to the animal receiving active hormone.

The solution used to control the experiments in which vasopressin was given was the same as that in which the hormone was diluted. This solution was also used as control for the experiments in which an 'aqueous' solution of oestrone was given. Arachis oil was used to control the experiments in which oil solutions of oestrone, progesterone, testosterone propionate, and desoxycorticosterone acetate were given, while 10% alcohol and distilled water were used as controls for the experiments in which 'Cortin' or 'Eucortone' were used.

Statement of results.

All results are expressed as a percentage of the weight of the animal at the start of the experiment.

EXPERIMENTAL RESULTS

No injections

The body-weight of uninjected axolotls (measured as described above) fluctuates very irregularly, within a range of $\pm 3\%$, during a 24-hour period. In twelve of our eighteen observations it rose at some time or other during the first 12 hours, and in all except one it had fallen below the initial weight by the 24th hour. The means of all the observations are plotted in Fig. 1.

Control injections

As a general rule the injection of arachis oil (maximum amount 1 c.c.) was followed by an immediate rise in body-weight, which within 4-6 hours had fallen below the initial weight, the changes in the weight-curve being fairly uniform in all of 23 experiments (Fig. 1). The initial rise, which usually showed itself in the first hour after the introduction of the oil, never exceeded 2·68%. It was rarely as much as 2%, and only in four cases could it not be accounted for by the weight of the oil itself. With four exceptions the final weight at the end of the 24-hour period of observa-

tion had not fallen below 3% of the first weight. The lowest weight was usually recorded at the 12th hour (see 'Note added in proof' at end of paper).

Comparison of the means of the pooled results, using Fisher's [1932] 't' test, shows that the weight changes which take place as a result of the injection of arachis oil are not at any time significantly different from
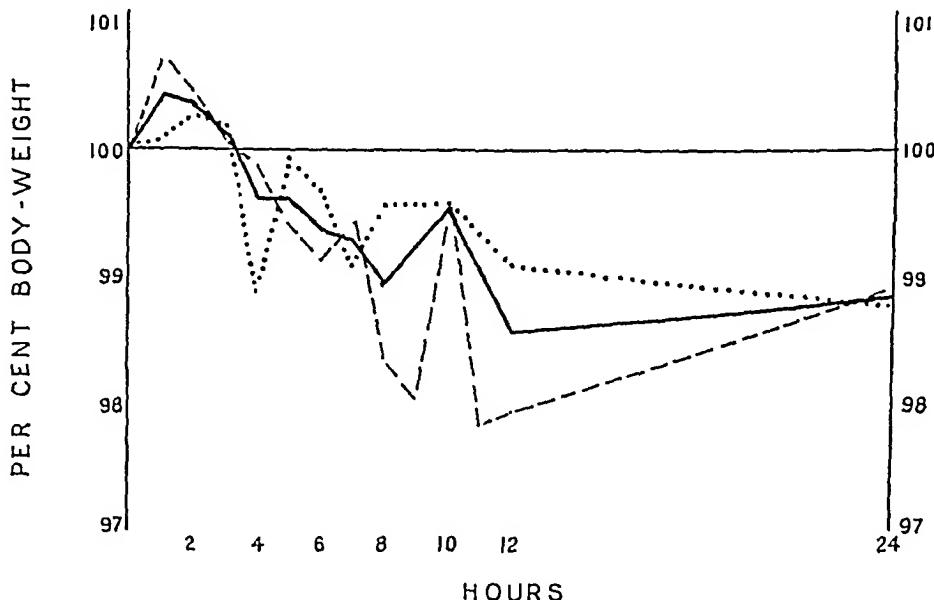
FIG. 1. Variation in weight of control axolotls during a period of 24 hours.
• • • • • • Mean of 18 observations on uninjected animals.
- - - - - Mean of 23 observations on animals injected with arachis oil.
————— Weighted-mean of observations on uninjected animals and on animals injected with arachis oil.

those which occur in an uninjected axolotl over a period of 24 hours, P in no case being even < 0.05. For this reason the means of the pooled results of both sets of these control observations have been used as a basis for calculating the effects of hormone injections, when administered in oil.

Both the injection of 10% alcohol and of the fluid that was used as a control for vasopressin and for 'aqueous oestrone' also led to a rise in body-weight, which was sustained longer than that occasioned by the injection of oil. As before, the gain in weight did not, as a rule, exceed 3% of the initial weight.

The injection of sterile water (0·5 c.c. in 6 experiments and 1 c.c. in 4 experiments) as a rule (but not always) led to a transient rise in body-weight. Body-weight in most cases had fallen below the initial level in 3 hours. The mean decrease in weight at the end of the 12th hour was 3·79%.

Vasopressin

Ten experiments were carried out with vasopressin, three animals being injected with 1·5 units in 0·75 c.c. of solvent, two with 1·3 units in 0·4 c.c., one with 0·66 units in 0·2 c.c., and four with 0·4 units in 0·4 c.c. In each experiment a control animal was injected with an equal amount of the solvent.

In every case the animals injected with the active hormone gained far more in weight than either the injected or the uninjected controls (Fig. 2), the increase in some of the experiments being as much as 9%. This result is in keeping with the results of all previous similar experiments on amphibia. The increase was greater on the higher than on the lower doses of the hormone, and the character of the response also varied according to the dose. In the experiments in which most hormone was given the animals continued to gain weight until at least the 12th hour, and body-weight had not returned to its initial level by the 24th hour. Animals given less than one unit of vasopressin as a rule began to lose weight after the 6th hour of the experiment, or even sooner, and by the 24th hour body-weight was well below the initial level (in one case by as much as 8·92%).

In any given experiment the chance that the weight-curve of a hormone-injected axolotl will rise above or fall below that of its control is 1 in 2. The probability that a rise above (or a fall below) the control curve could occur by chance in each of n consecutive experiments is 1 in 2^n. Taking the usual statistical criterion of significance, namely, that there is less than 1 chance in 50 of an observed relation occurring fortuitously, it is clear that 6 or more consecutive similar results in these experiments constitute evidence for a significant change. The experimental results described above are thus evidence of real physiological effects.

That the effects were significant was also established by pooling all the observations made on animals injected with vasopressin, and comparing the means, by Fisher's [1932] 't' test, with the means of all the observations made on animals that were given either the control solution or no injection at all. When using this test, the more rigid criterion of significance, $P < 0.01$, was chosen in preference to $P < 0.02$. At the 6th hour the results of the comparison of the mean of the vasopressin-injected animals with that of the control-solution-injected animals were: $n = 14$, $t = 3.6$, and $P < 0.01$; the corresponding figures in the comparison with the uninjected controls were $n = 19$, $t = 6.1$, and $P < 0.01$.

Oestrone

Nine experiments were performed in which oestrone was administered in oil, three animals receiving 1 mg. in 1 c.c. of solvent, one 500 μg. in 0·5 c.c., and five 400 μg. in 0·4 c.c. The experiments were controlled in

the usual way by injecting other animals with an equal amount of the oil solvent.

The results were uniform in so far as in every case the experimental
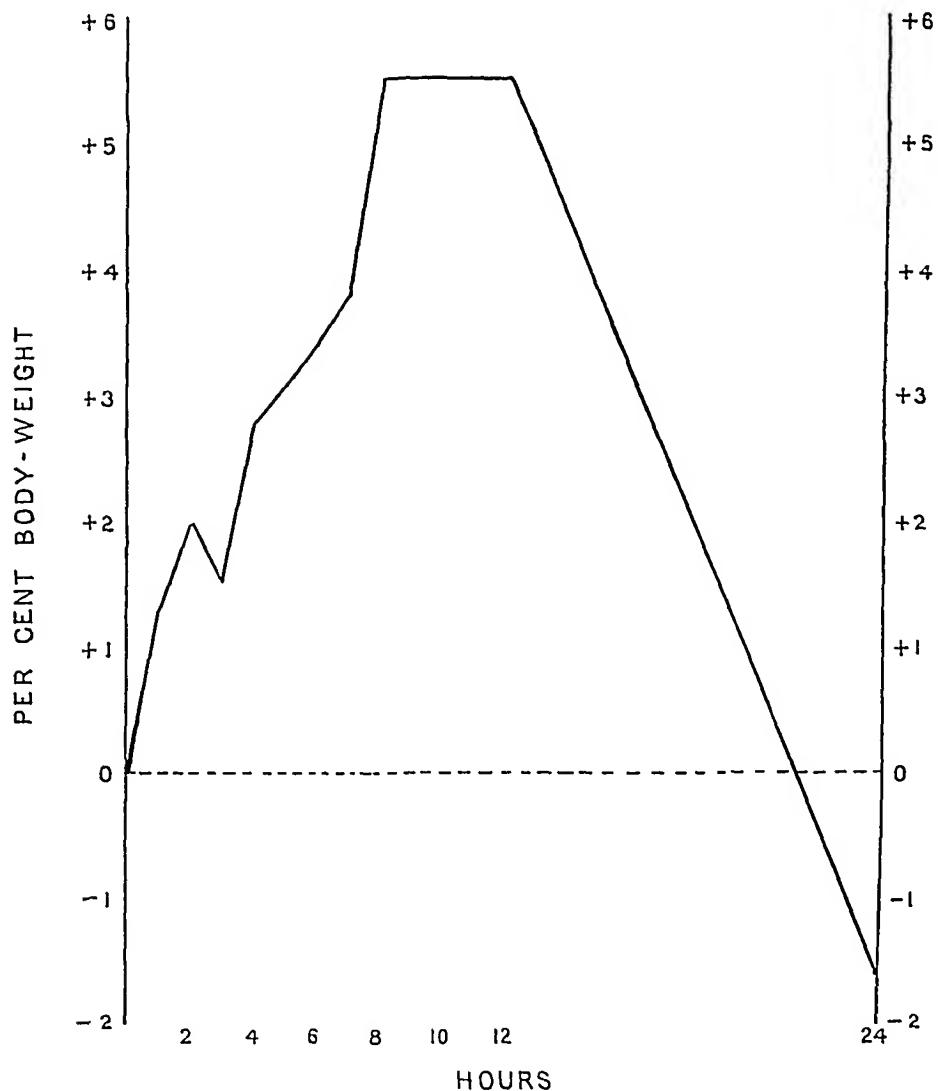
FIG. 2. Effect of vasopressin on body-weight of axolotls. The points plotted are the differences between the mean observations on 10 animals injected with the hormone, and the mean observations on axolotls which received the control fluid.

animals put on more weight than the injected controls (Fig. 3). The effect was less marked in the experiments in which only 400 μg. of oestrone were given, and in all the experiments it was considerably less than the changes observed when high doses of vasopressin were administered. The maximum rise recorded in the oestrone experiment was 5·58%.

The weight changes under the influence of oestrogenic stimulation were of a fairly uniform character, an immediate rise in the first 2–3 hours being followed by a fairly regular and slow fall. In this respect the changes differed from the irregular ones which were observed over a 24-hour period in uninjected animals, although the magnitude of the change observed in some of the latter cases was little different from the changes observed in the oestrone experiments. Comparison of the means of the pooled
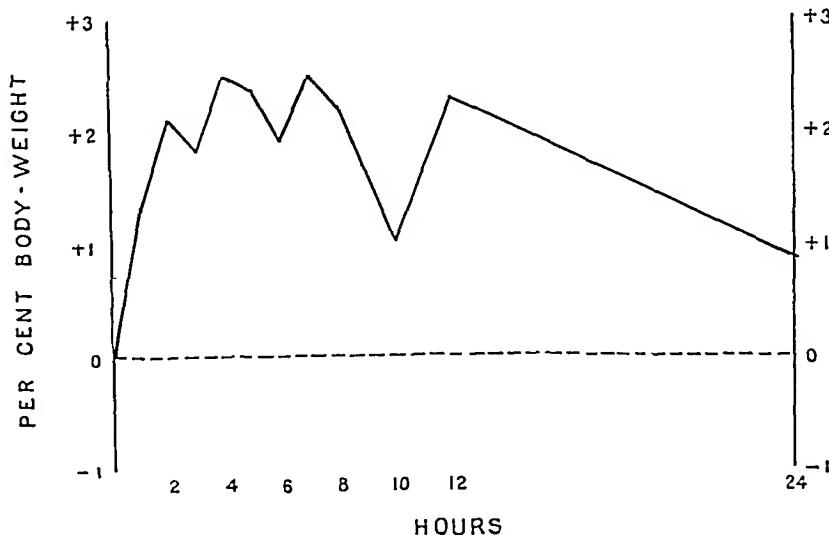
Fig. 3. Effect of oestrone on body-weight of axolotls. The points plotted are the differences between the mean observations on 9 animals injected with oestrone in oil, and the weighted-means of both sets of data plotted in Fig. 1.

results shows, however, that the weight changes which occurred in the oestrone-injected animals were significantly greater than those which took place in normal axolotls. Thus at the end of the second hour $t = 4\cdot36$, $n = 23$, and $P < 0\cdot01$. The corresponding figures in the comparison of the pooled oestrone results with those for the control oil-injected animals were $t = 3\cdot85$, $n = 28$, and $P < 0\cdot01$.

Five experiments were carried out using an aqueous solution of oestrone. One animal received 30 μg. in 1·2 c.c. of the solvent described on p. 388, the total volume being administered in three injections over a short period. Another animal received 20 μg. in 0·8 c.c. of solvent in two injections, and three animals were given 10 μg. in 0·4 c.c. in a single injection. The usual control experiments were made with solvent alone. The results obtained suggest that oestrone in the small amounts administered has practically no effect on body-weight.

Progesterone

Eleven experiments were performed with progesterone. Seven animals were given 10 mg. in 1 c.c. of oil, one 8 mg. in 0·8 c.c. of oil, one 6 mg. in 0·6 c.c. of oil, and two 2 mg. in 0·2 c.c. of oil. With the exception of the one experiment in which 6 mg. were injected, all the animals injected with hormone showed a much greater increase in weight than the controls which received oil alone (Fig. 4). The general character of the response
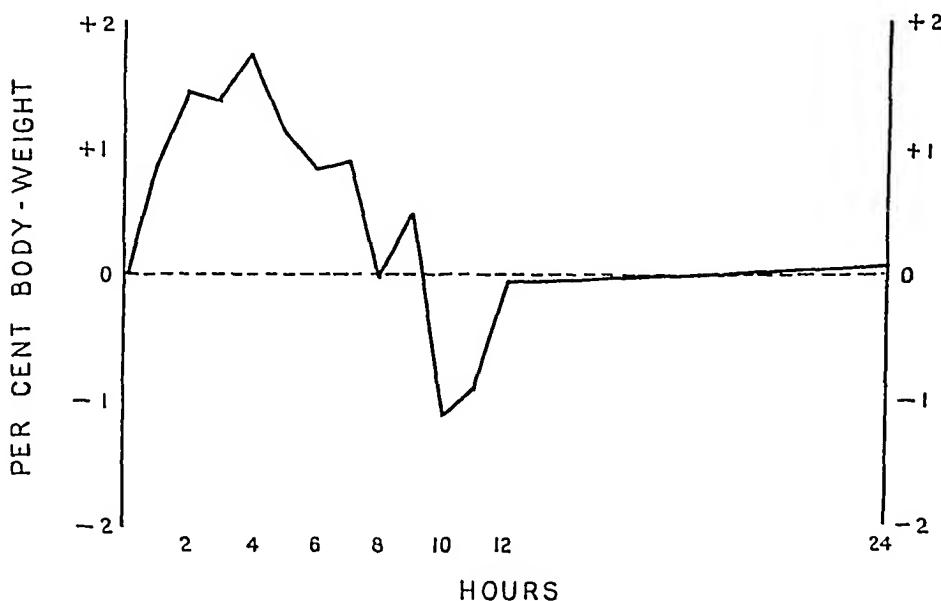
FIG. 4. Effect of progesterone on body-weight of axolotls. The points plotted are the differences between the mean observations on 11 animals injected with progesterone in oil, and the weighted-means of both sets of data plotted in Fig. 1.

was the same as that of the oestrone experiments, the rise in weight occurring in the first 2 hours of the experiment and being maintained longer in the animals that were given the larger doses of hormone.

Analysis showed that the rise in weight was statistically significant. Thus the following figures were obtained in the comparison of the mean of the pooled results for progesterone at the end of the 2nd hour with the corresponding mean for the uninjected controls: $t = 3·56$, $n = 23$, and $P < 0·01$. Corresponding figures in the comparison of the pooled results with those for the control oil-injected axolotls were $t = 2·86$, $n = 28$, and $P < 0·01$.

Testosterone propionate

Ten axolotls were injected with testosterone propionate. One was given 50 mg. in 1 c.c. of oil, three were given 40 mg. in 0·8 c.c. of oil, and six 25 mg. in 0·5 c.c. of oil. Only one of the ten hormone-injected animals

failed to put on more weight than the controls. The general character of the response was similar to that to oestrone (Fig. 5), and statistical analysis showed that the rise in weight was significant. Thus comparison of the mean of the pooled results at the end of the 2nd hour with that of the uninjected controls gave the following results: $t = 3.31$, $n = 24$, and $P < 0.01$. The corresponding figures in the comparison with the means of the pooled results for the oil-injected controls were $t = 2.94$, $n = 29$, and $P < 0.01$.
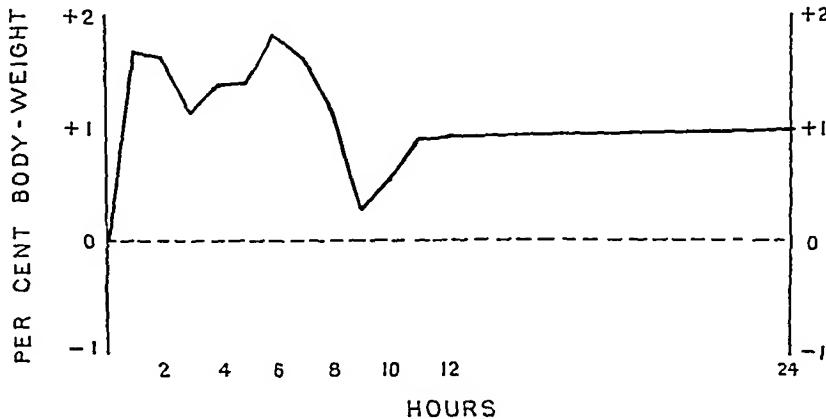
FIG. 5. Effect of testosterone propionate on body-weight of axolotls. The points plotted are the differences between the mean observations on 10 animals injected with testosterone propionate in oil, and the weighted-means of both sets of data plotted in Fig. 1.

Adrenal cortical hormone

Sixteen experiments were carried out with adrenal cortical hormone. Eight animals were given 5 mg. of desoxycorticosterone acetate in 1 c.c. of oil, one was given 2·5 mg. of the acetate in 0·5 c.c. of oil, and two were injected with 2 mg. of the same substance in 0·4 c.c. of oil. The Upjohn 'Cortin' preparation was used in three experiments, the equivalent of 32 g. of adrenal tissue in 0·8 c.c. of solvent being given to one, and of 20 g. in 0·5 c.c. to two animals. Two animals were also given the Allen & Hanbury 'Eucortone' preparation, both receiving the equivalent of 37·5 g. of cortical tissue in 0·5 c.c. of solvent. Arachis oil was used to control the desoxycorticosterone acetate experiments, 10% alcohol to control the Upjohn preparation of 'Cortin', and sterile water the two experiments in which the Allen & Hanbury 'Eucortone' was injected.

The results of the three experiments in which the Upjohn 'Cortin' was administered were very striking: an immediate rise in weight, which could be accounted for by the weight of the injected substance, being quickly

followed (at the end of the 1st hour) by a sharp fall (Fig. 6). In all three cases the weights of the hormone-injected animals fell far below that of
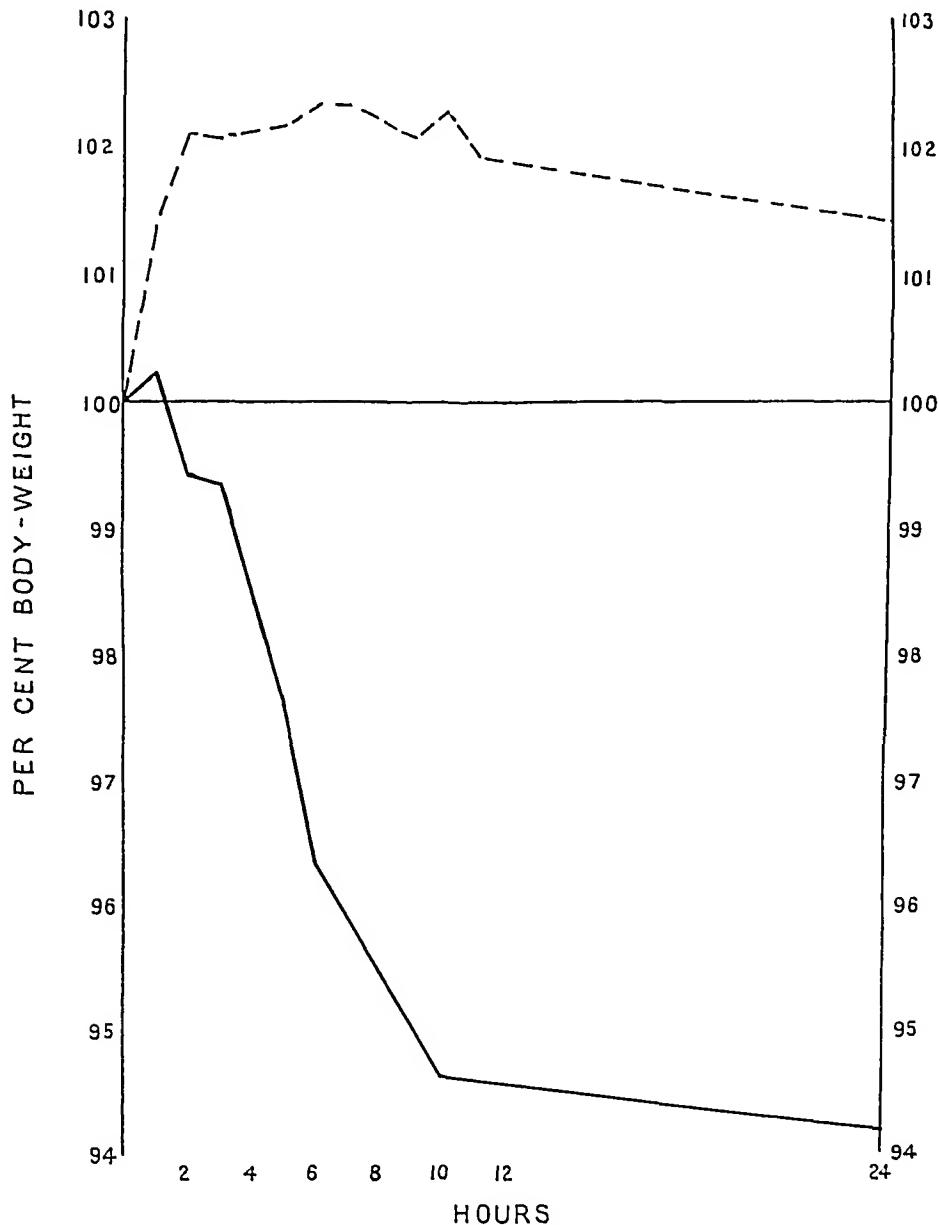
FIG. 6. Effect of 'Cortin' on body-weight of axolotls.
———— Mean of observations on two animals injected with 0·5 c.c. of 'Cortin'.
- - - - - Mean of observations on three animals injected with 0·5 c.c. of 10 per cent. alcohol.

the controls. The results were not clear-cut in the remaining experiments. In one of the two experiments in which 'Eucortone' was injected body-

weight fell below, and in the other rose above, that of the controls into which sterile water was injected. Similarly, while the first experiments in which desoxycorticosterone acetate was given also demonstrated a fall in the weight of the hormone-injected as compared with the control animals, this conclusion was not fully borne out in later experiments. For although the mean weight-curve of the animals which were given the
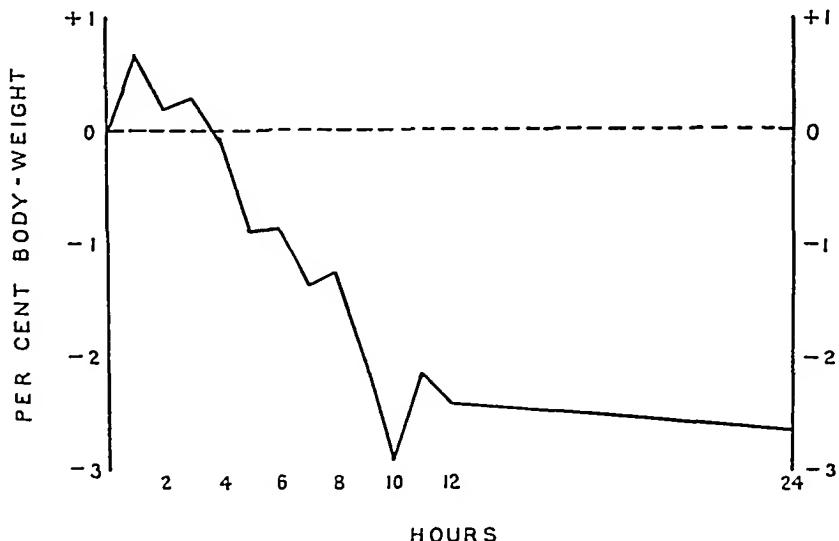
FIG. 7. Effect of desoxycorticosterone acetate on body-weight of axolotls. The points plotted are the differences between the mean observations on 11 animals injected with the acetate in oil solution, and the weighted-means of both sets of data plotted in Fig. 1.

acetate falls below the mean weight-curve of all the control observations (Fig. 7), statistical analysis indicates that the differences between the two are not significant (but see 'Note added in proof' at end of paper). Nevertheless it is of considerable interest that body-weight in axolotls tends to fall as a result of the injection of adrenal cortical hormone. In so far as changes in weight indicate the retention or loss of water, this finding suggests that cortical hormone acts very differently in mammals and axolotls.

SUMMARY

Adequate amounts of vasopressin, oestrone, progesterone, and testosterone propionate cause a significant rise in the body-weight of axolotls. The effects of adrenal cortical hormone are somewhat variable, but as a rule it causes a fall in body-weight as compared with controls. On the basis of previous work, these changes may be attributed to changes in body-water.

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Note added in proof

The results of 8 further control experiments, carried out when the paper was going through the press, agreed very closely with those reported above, p. 389. Four axolotls were given 1 c.c. and four 0·5 c.c. of arachis oil. The mean relative weights at hourly intervals after injection did not differ significantly from the previously obtained values, although in 5 further cases (making 9 exceptions in a total of 31 control experiments) the final weight at the end of the 24-hour period of observation had fallen below 3% of the first weight.

These new observations were made as controls for 11 additional experiments in which desoxycorticosterone acetate was administered. Seven axolotls were given 5 mg. in 1 c.c. of oil and four 5 mg. in 0·5 c.c. of oil. The results did not differ significantly from those obtained in the first group of desoxycorticosterone experiments. Both sets of figures were therefore pooled, and compared with the pooled results of all the control experiments in which oil alone was given (31 in all). The results of this more extensive analysis showed that at the 24th hour the desoxycorticosterone acetate had caused a significant fall in weight ($n = 48$, $t = 4·23$, $P = < 0·01$). The figures for the pooled desoxycorticosterone experiments were also compared with those for the uninjected controls, and it was found that in this case too the weight of the injected animals was significantly lower at the 12th and 24th hours ($n = 32$, $t = 3·14$, $P = < 0·01$, and $n = 42$, $t = 5·05$, and $P = < 0·01$ respectively).

CONTRIBUTIONS TO RESEARCH ON THE FEMALE SEX HORMONES

THE IMPLANTATION OF THE MOUSE EGG

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From the Zoological Institute of the University of Basle

(*Received 9 October 1939*)

WHEREAS the influence of the follicular hormone on the oestrous phenomena and the cyclical changes in the female genital tract, and the provocation of the progestational phase of the endometrium by the corpus luteum hormone, seem to be perfectly clear, the function of the corpus luteum in the process of implantation of the egg has not yet been explained. This most important process, which can easily be regarded as the purpose and fulfilment of the whole genital function, is not entirely understood in its interactions of cause and effect.

The corpus luteum 'protects the egg' [Fraenkel, 1903, 1910], it directs the implantation, and its presence is indispensable for the accomplishment of the implantation [Bouin and Ancel, 1910; Ancel and Bouin, 1924; Corner and Allen, 1929; Courrier, 1935; Courrier and Kehl, 1938; Pincus and Werthessen, 1938, and others]. According to Corner [1928] the corpus luteum is also necessary for the nutrition of the blastocyst until implantation takes place. But we do not know in what way the corpus luteum hormone affects the process of implantation, i.e. if the stimulating of the progestational phase of the uterine mucosa only means the providing of a suitable environment for the fertilized egg, if the transformed endometrium acts upon the egg and stimulates it to implantation, or if the corpus luteum stimulates the egg directly to implant itself.

The present paper is the first of a series of publications the object of which is to contribute to the elucidation of various sides of the problem of implantation. This first part concerns the realization of implantation, and the part played by the corpus luteum hormone in this process. The lack of knowledge regarding the latter is obvious from the fact that in his monograph on *The Eggs of Mammals*, Pincus [1936] quotes either excessive or insufficient secretion of corpus luteum hormone (that is to say radically opposite conditions) as possibly the cause of delayed implantation, produced physiologically or experimentally.

Among the authors who have described the process of implantation we find one group which considers the egg as the active agent facing the passive endometrium, whereas another group holds the opposite opinion.

The representatives of the first view consider the egg as an agent intruding actively into the mucosa and forcing its way into the uterine wall by enzymatically destroying the epithelium. Spee [1901], an adherent of this theory, describes pseudopodia-like protuberances of the implantation pole of the guinea-pig egg, which penetrate into the epithelium. The blastocyst enters through the chemically dissolved epithelium into the mucosa which is afterwards also destroyed. Sansom and Hill [1930], who fully confirm the observations of Spee, have also observed these pseudopodia and the dissolution of the epithelium by cytolysis. According to these investigators, however, the sub-epithelial tissue is not destroyed. They doubt whether the protuberances are actually a secretion or the pseudopodia described by Spee. Spee recognizes in their pictures the pseudopodia formerly described by him and formulates the process drastically: 'the germ forms roots'.

An active part is also attributed to the egg by Assheton [1895] and Schoenfeld [1903], who explain the mechanism of implantation by physical activity, namely, by the hydrostatic pressure in the blastocyst cavity leading to penetration of the egg into the uterine wall at the point where it presents the least resistance.

The opinions of Huber [1915] and Kirkham [1916] indirectly support the theory which considers the uterine wall as playing the active part. They indeed speak of the reaction of the mucosa on the egg, thus attributing the initial action to the egg, yet they consider the reaction of the mucosa as the only possible means given to the egg to become implanted.

The provocation of pseudopregnancy by sterile coitus [Bouin and Ancel, 1910], and the experimental production of deciduomata by mere mechanical stimulation of the sensitized mucosa [Loeb, 1907, 1908, 1909] demonstrate the power of the uterine wall to form decidual tissue without the presence of an egg. These facts are the basis of the opinion which considers the uterus as playing the active part in provoking the gestational changes in the mucosa. According to Mossman [1937] the blastocyst does not actively destroy the epithelium, the stimulus of the egg being non-specific.

The uncertainty of the differing opinions often leads to contradiction. For instance, Goetz [1937, p. 286] speaks clearly of a hypertrophy of the mucosa produced by local induction by the blastocyst at the implantation sites, but in contradiction he finds regularly spaced, local alterations in a uterus in which there are no blastocysts at all, and which he considers to be the same formations as those observed in the hypertrophied implantation sites.

In this connexion we might also quote an observation made by Sansom and Hill [1930], the explanation of which is in contradiction to their attributing the active part to the egg. Spee speaks of cases of double

implantation in the guinea-pig, in which the egg comes into contact with the uterine wall in two places. He supposes that this, perhaps, happens regularly in the mouse, which is indeed the case, the *Muridae* showing the so-called eccentric mode of implantation (cf. Plate I, Fig. 1). Sansom and Hill describe in the guinea-pig egg two cases similar to those called double implantation by Spee, but they give the explanation that the blastocyst somehow got into contact with the opposite uterine wall and that by this contact or approach the trophoblast is induced to react, which would not otherwise have occurred; for after implantation has occurred, this part of the trophoblast (ectoplacental trophoblast) lies directly touching the epithelium, yet does not react in any way. This explanation doubtless assumes that the uterine epithelium takes an active part as inductor of the blastocyst, producing a reaction of the trophoblast following the stimulation by the epithelium.

Mossman [1937] summarizes the complex character of the process of nidation by calling it 'a set of interactions between the embryo and the maternal organism'.

One fact may be considered as absolutely certain, that is the necessity of the presence of corpus luteum hormone for the formation of the pro-gestational endometrium, as well as for the accomplishment of the im-plantation and the first stages of development of the implanted egg. We might say that the presence of this hormone is indispensable to both participants in the process. But we do not know exactly how it acts.

Another phenomenon which has always interested investigators and has so far not been explained is the regular spacing of the ova in the uterus of the mammalian species having several young to a litter [Huber, 1915; Keye, 1923; Mossman, 1937; Parker, 1931; Sobotta, 1901; Widakowich, 1911]. We do not know anything definite about the mode of migration or transport of the ova down the uterine cornua, whether it is effected by activity of the germ, chemotaxis, peristalsis [Keye, 1923], or ciliary movement [Mandl, 1908]. Goetz [1937] has also observed cilia in the uterine epithelium of *Hemicentetes*. But even if we knew by which means the ova proceed, this would not explain the reason for the arrest of the blastocysts and their regularly spaced implantation.

Different suggestions have been made to explain this phenomenon, for instance that the blastocyst attains some sort of physiological relation with the mucosa which then renders the immediate neighbourhood refrac-tory to any other embryo [Mossman, 1937], or that an acceleration in the rate of uterine growth around the site of implantation creates for each embryo a segment of uterus to nourish it, thus showing an appearance of uniform spacing [Hammond].

But Mossman's suggestion has no experimental proof, and Hammond's

observation does not explain the regular spacing of the ova actually established before implantation takes place.

Let us examine exactly the morphological characteristics which can be observed in the mouse endometrium before and during implantation.

Ovulation takes place in the late oestrous stage. The tubal migration of the ova, during which stage maturation and insemination occur, takes about 4–5 days. The eggs, entering the uterus in a clump, at once disperse and begin to pass rapidly through the uterine horn along its whole length and finally become fixed at equal distances from each other at the sites of their later implantation [Sobotta, 1901; Burckhard, 1901].

In cases of physiologically delayed implantation, e.g. in rats and mice which have been copulating at the post-partum ovulation and which rear their preceding litter, the pre-implantation vesicles lie free for many days in the uterine cavity at the places of their later nidation. During this time no implantation occurs, that is to say, there is no reaction either by the mucosa or by the blastocyst. This phenomenon is to be examined in the following paper, the relevant experiments having already been carried out.

Implantation occurs in a species-specific way; either on the mesometrial side of the uterus as in *Tarsiidae*, or on the anti-mesometrial side as in *Muridae, Caviidae, Leporidae,* and *Sciuridae* [Mossman, 1937], or in certain species 'orthomesometrially' (i.e. alternating ventrally and dorsally) as in *Centetes* and *Hemicentetes* [Goetz 1937].

MATERIAL AND METHODS

A great number of uteri of the albino mouse presumably containing pre-implantation blastocysts have been prepared for histological examination. Transverse sections 5–10μ thick were made. The animals were killed with chloroform and at once dissected and the whole genital tract fixed in various solutions, among others Flemming's fluid being used. This proved to be very important. As far as I can see most of the previous investigators employed solutions such as Bouin's or Zenker's fluid, formalin, or alcohol. But after the use of a solution containing osmic acid a secretion is seen in the sections which cannot be seen when using other means of fixation.

The method of determining the presence of spermatozoa in the vaginal smear in order to time the matings was found preferable to the method of looking for the vaginal plug. Although the plug is formed after nearly every mating (Lewis and Wright [1935] found only one case among 103 copulations observed where it was not formed), it is very often possible that it will not be observed, probably because most matings occur during the night and the plug seems often to be lost very soon afterwards. Long

and Evans [1922] in their investigations on the rat succeeded in finding the plug only in 55% of the established pregnancies. On the other hand, taking twice daily the vaginal smear of the females in heat who have the opportunity of mating we shall most certainly find the spermatozoa in it. The smear is spread on a glass slide, stained with haematoxylin and eosin without preceding fixation, and dried between two sheets of blotting-paper.

DESCRIPTION OF THE HISTOLOGICAL ASPECT OF THE PROGESTATIONAL ENDOMETRIUM

While previous authors such as Burckhard [1901] or Widakowich do not note any changes of the uterine mucosa of the mouse until the implantation of the ova has taken place, other investigators give the following descriptions of the changes appearing during the metoestrous stage, these changes being the same with or without following pregnancy.

E. Allen [1922] indicates: fading of the basement membrane of the epithelium, small vacuoles in and among the epithelial cells, advancing of the epithelial nuclei towards the uterine lumen causing the formation of a broad red- or pinkish-staining band behind the nuclei, and leucocytic infiltration of the tissue beginning in this pinkish band, which for that reason is considered by Allen as a primary manifestation of degeneration provoking leucocytosis.

Clauberg [1930, 1931] observes almost the same changes: increase in density of the stroma with enlargement of its cells and nuclei, advancing of the epithelial nuclei from the base to the centre of the cells, the pink-staining band, fading of the basement membrane and the cell outlines on the basal side of the nuclei, but no leucocytic infiltration into the pink band. Therefore Clauberg does not consider the pink band as a phenomenon of degeneration, but as the beginning of the symplasma-like fusion of the epithelium' which prepares the formation of the gestational mucosa. In contradiction to Allen, who ascribes no primary causative function to the corpus luteum in the mouse when no fertilization takes place, Clauberg considers these changes as produced by the corpus luteum, but considers the luteal phase of very short duration in the mouse. He succeeded in provoking these changes artificially by injecting corpus luteum extracts into ovariectomized mice which had been treated previously with follicular hormone.

Corner and Allen [1929] had already provoked the same changes in the rabbit; the test elaborated by them on the rabbit uterus is now generally employed as well as the 'uterine lace' test described by Bouin and Ancel [1910].

Clauberg [1930] tried to use the mouse for tests instead of the rabbit, but found the uterine changes far less obvious and less conspicuous

in the mouse endometrium. W. M. Allen [1931] tried the same test on the rat. He describes an epithelium with a smooth cytoplasm, granulated only at the base, small nuclei in the centre of the cells, indistinct cell outlines and basement membrane, syncytial stroma and epithelium. Allen characterizes the stroma as presenting the aspect of growth, the epithelium as presenting that of secretion, without, however, exactly describing this secretion.

It can be stated in general that for the luteal phase in the mouse and rat, which corresponds to the early pregnancy stage in the rabbit and to the secretion phase in man, nowhere do we find a real description of a secretion. This is partly explained by the fact that in the mouse endometrium the uterine glands do not undergo the remarkable growth and ramification of the alveoli which so conspicuously alters the aspect of the rabbit endometrium (uterine lace).

Now we will consider the histological aspect of a transverse section through a uterus with a blastocyst ready for implantation, fixed in Flemming's fluid (Plate I, Figs. 1 and 2; Plate II, Fig. 3). These figures show an epithelium doubtless in most active secretion. The basement membrane is very well preserved, the cell borders are indistinct, but not entirely lacking, the nuclei are somewhat advanced towards the lumen, though not farther than to the centre of the cells, and some of them are still nearer to the base. The epithelium is slightly flattened in the neighbourhood of the blastocyst, as described by Sobotta [1901]. Radiating from the nuclei we find, especially at the basal and the opposite end of them, a dense conglomeration of granular secretion, forming a conspicuous black ribbon in the plasma of the epithelial cells (Plate II, Fig. 3). In the cells in which the section did not touch the nucleus, or went only through a small segment of it, the secretion granules fill the whole body of the cell, showing that the granular secretion completely surrounds the nuclei. The same secretion appears, principally basally, in the epithelium of the uterine glands (Plate III, Fig. 7).

The stroma shows decidual changes, a syncytial cytoplasm, great round nuclei, and many capillaries.

The epithelial secretion, which appears only in the sections of uteri preserved in Flemming's fluid, is present only in the uteri containing blastocysts ready for implantation. It appeared faintly in a uterus which contained no blastocysts; further investigation, however, led to detection of the ova in the oviduct, and the epithelium of the oviduct also showed the presence of the secretion. It is, therefore, without doubt, a secretion connected with the presence of the blastocyst and proving the preparation of the endometrium for the reception of the egg. This preparatory stage, the secretion stage, is the corpus luteum phase during which the epithelium

is under the influence of this gland. This corpus luteum of early pregnancy is apparently different; it is perhaps stronger functionally, although not morphologically, than the corpus luteum of the genital cycle unaffected by pregnancy. (E. Allen finds no difference during the first four days.) In the uteri containing no eggs the corpus luteum indeed never provoked the secretion. It might, however, be that it existed for a short time, but disappeared so soon again that it could not be detected.

In some places (Plate I, Fig. 1) the secretion seems to migrate into the cells of the blastocyst. As, according to Corner (1928), the corpus luteum secretion is necessary for the nutrition of the egg until it becomes implanted, this observation, as well as the presence of the secretion in the oviduct epithelium, can easily be explained in this way. This interpretation is confirmed by the following observation. Figs. 4, 5, and 6 (Plate II) represent transverse sections through a uterine horn containing a series of embryos already implanted. We can see that the secretion diminishes and vanishes wherever the sections approach the implanted embryo (Figs. 4 and 5 are of sections which border on the zone containing the blastocyst). Between the implantation sites the secretion is still visible, and that only on the mesometrial side of the horn (Plate II, Fig. 6). This rhythm is repeated for every germ. We thus have an epithelial tube in which the secretion, which had been present in the whole length before implantation, has vanished in and around the implantation sites, but is still preserved in between these zones on the mesometrial side of the tube, that is to say, on the side where implantation does not occur. The secretion seems therefore to have been exhausted by the implantation and absorbed by the blastocyst.

In Plate III, Fig. 7, representing a section of a horn in which the secretion has been experimentally produced (see below) and is much stronger, we see that it is more intense on the anti-mesometrial side of the lumen than on the mesometrial. (E. Allen finds that along the line of attachment of the uterine ligament there are no uterine glands; I can fully confirm this observation.) The fact that the secretion is stronger on the anti-mesometrial side may mean in the first place greater readiness of this side to receive the ovum, but it might also be taken as the cause provoking implantation on this side. We may assume that the eggs are chemotactically attracted by the zone presenting the intense secretion, the latter thus causing their attachment to the mucosa. This would be a proof that the stimulus for implantation is exercised by the secreting epithelium, that is to say, that the uterine wall plays the active, the ovum the passive part among the factors which cause implantation. This hypothesis does not, however, exclude the possibility that the action of the epithelium is followed by a reaction of the blastocyst and that, after

having penetrated into the epithelium, the embryo itself acts on the mucosa.

There is another observation which allows a suggestion to be made concerning the spacing of the blastocysts in the uterus. Looking through the series of sections we note a distinct rhythm in the intensity of the secretion along the length of the horn. Alternating with a section showing a stronger secretion on the anti-mesometrial side we find one showing an equal secretion all round the lumen (Plate III, Figs. 7–12). The sections have been chosen at intervals out of the series, not, however, at mathematically equal distances. We may assume that the anti-mesometrial areas of intense secretion correspond to the implantation sites, i.e. that in these places the migrating blastocysts come to a rest at the place of their later implantation. In cases of delayed implantation where the blastocysts lie free in the lumen (see above) the secretion is indeed stronger in the neighbourhood of the egg, but it has not yet migrated through the epithelium. It is mostly accumulated behind the nuclei as in Plate III, Fig. 7, whereas in the epithelial cells of the uteri containing blastocysts just becoming implanted the secretion is equally strong on both sides of the epithelial nuclei (Plate I, Figs. 1 and 2), or even stronger on the side of the lumen.

We cannot assume that the zones of intense secretion correspond numerically to the implantation sites, as, according to the findings of Merton [1937], the number of eggs in the oviduct does not correspond to the number of the new-born young, which means that the number of embryos of two successive litters is not the same in one uterine horn; but we can take it that not every one of a series of pre-formed areas is utilized for implantation.

The Figs. 7–12 (Plate III) represent sections through a uterine horn containing no blastocysts, but in which the corpus luteum phase had been provoked by injections of synthetic corpus luteum hormone, a proof that the secretion is indeed produced by the activity of the corpus luteum. By using this injection method it will most probably be possible to employ the mouse uterus as a test object for progesterone. This will be the subject of a later paper.

Summary

Transverse sections through the mouse uterus containing blastocysts ready for implantation, fixed with Flemming's fluid, show an intense secretion radiating from the nuclei of the epithelial cells.

This secretion is interpreted as the principal criterion of the corpus luteum phase in the mouse endometrium. It is only to be found when pre-implantation blastocysts are present in the uterus; it is therefore pro-

duced by the corpus luteum of early pregnancy which is different from the corpus luteum of the genital cycle. It demonstrates the active part played by the endometrium in the process of implantation.

It is assumed that the secretion is absorbed by the blastocyst, thus providing its first nutrition.

The secretion can be produced, when no eggs are present, by injections of synthetic corpus luteum hormone. It is more intense on the anti-mesometrial side of the uterine tube than on the mesometrial. It is also rhythmically increasing and decreasing along the length of the uterine horn. It is therefore suggested that the areas of strong secretion are chemotactically attracting the blastocysts, thus causing their implantation in these places.

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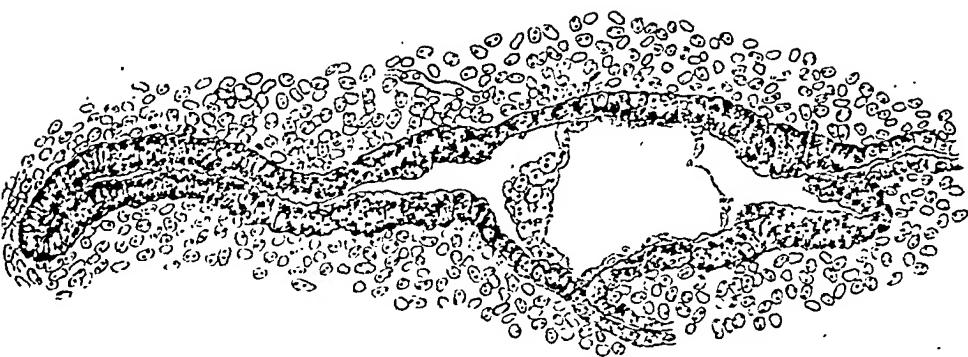
Fig. 1. Transverse section through a uterine horn containing a blastocyst ready for implantation. In the stroma only the nuclei have been drawn
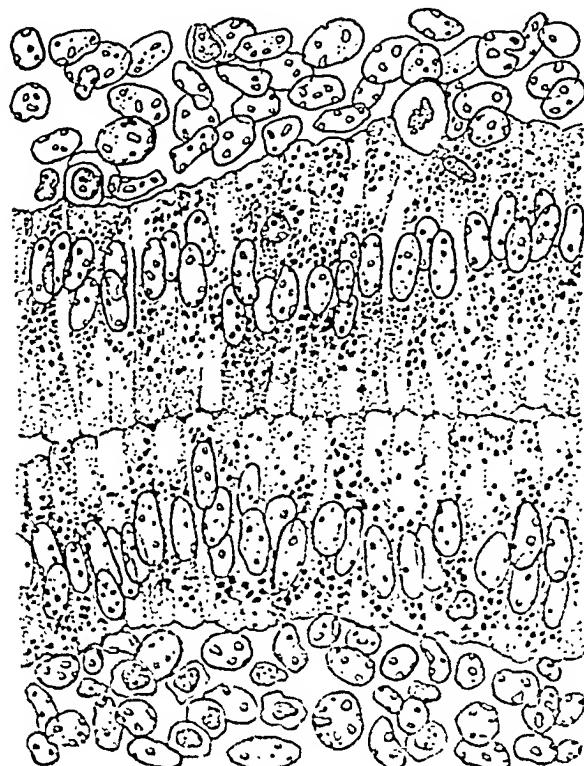
Fig. 2. Part of the uterine epithelium showing the granular nuclei of the epithelial cells. In the stroma only the nuclei have been drawn

FIG. 3. Transverse section through a uterine horn showing the secretion in the epithelium. × 52
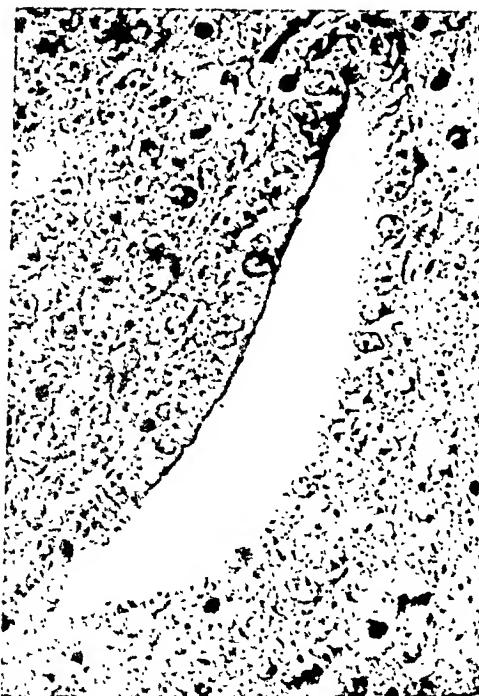
FIG. 4. Transverse section through the zone of the uterine horn bordering on the implantation site. No secretion is to be seen in the epithelium. × 288

FIG. 5. Transverse section through the zone of the uterine horn bordering on the implantation site. o secretion is to be seen in the epithelium. × 288

FIG. 6. Transverse section through a uterine horn showing the epithelial secretion on the mesometrial side (above) only. × 52
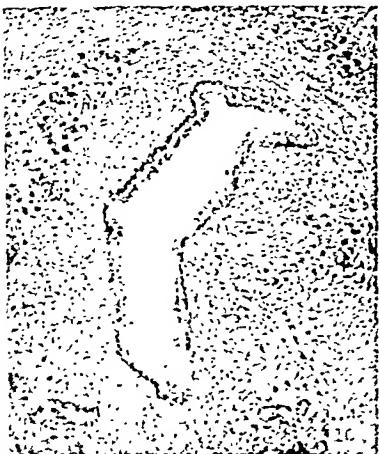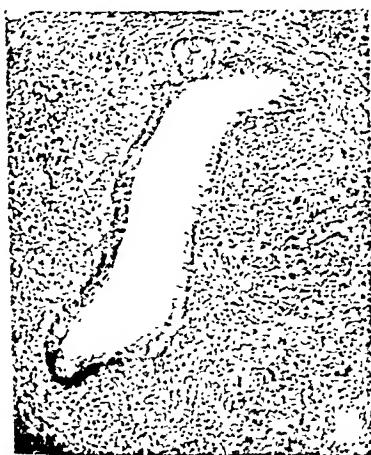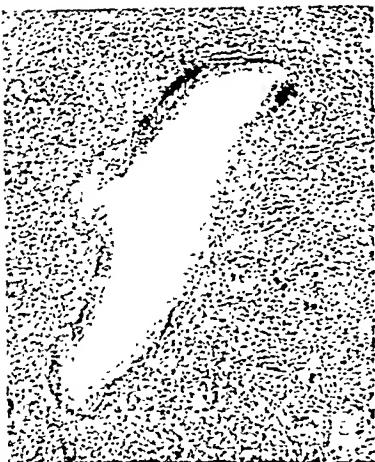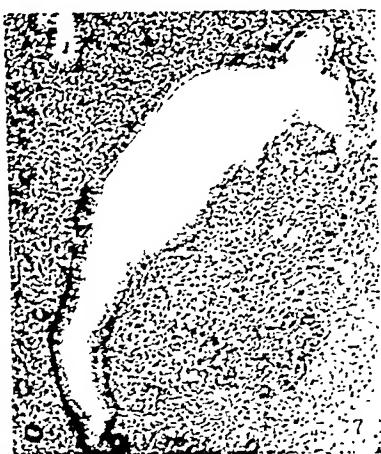
Figs. 7-12. Transverse sections through a uterine horn three weeks after the operation.

GROWTH OF THE REPRODUCTIVE AND ENDOCRINE ORGANS OF THE FEMALE RABBIT

By C. W. EMMENS

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IN a previous communication from this laboratory, Deanesly and Row-lands [1936] analysed the growth of the reproductive and endocrine organs of male and female guinea-pigs. A similar investigation has now been made on the female rabbit, for which little information appears to be available. Brown, Pearce, and van Allen [1926a, b] analysed the organ-weights of apparently normal rabbits with reference to effects that might be produced by obscure lesions, but their data are not given in a form which enables any conclusions about the relation of organ- and body-weights to be drawn. Robb [1928, 1929] and Allanson [1932] investigated the growth of the pituitary body of male and female rabbits and agreed that its weight bears a constant relationship to that of the cleaned carcass.

Material and Methods

Animals. Eighty-three virgin female rabbits of from 280 to 2,640 g. gross body-weight were used. Forty-one were Dutch-marked, and forty-two were of the Himalayan breed, all from the colonies maintained at the Institute's Farm Laboratories.

Preservation and weighing. The organs, except the pituitary glands, were fixed in Bouin's fluid immediately after removal, left for 24 hours and then placed in 70% alcohol for a similar period before weighing. The pituitary glands, not all of which were kept, were placed directly in acetone, since they were needed for other purposes, and weighed about 2 days later. Weighing was done on a torsion balance, accurate to within ±1 mg., except in the case of some of the heavier uteri, which were weighed on an ordinary chemical balance.

Treatment of results. The results are presented in the form of scatter-diagrams. It has not been thought profitable to calculate regression lines or to convert the data into logarithmic forms, although the organ-weights frequently appear to bear a linear or simple curvilinear relation to body-weight. A distinction has been maintained in the diagrams between Dutch-marked and Himalayan rabbits, since, in the case of the adrenal glands and uteri, there is evidence that the two breeds differ. Dutch rabbits are connoted in the diagrams by black dots, and Himalayan rabbits

by open circles. When considering the ovaries and uteri, the occasional rabbits which were found to have ovulated are connoted by black squares for the Dutch rabbits, and unshaded squares, or a dotted circle, for the Himalayan rabbits.

Ovaries

Fig. 1 shows the distribution of ovary-weights. At a body-weight of below 750 g. the ovaries remain small, weighing 30 mg. or less. From
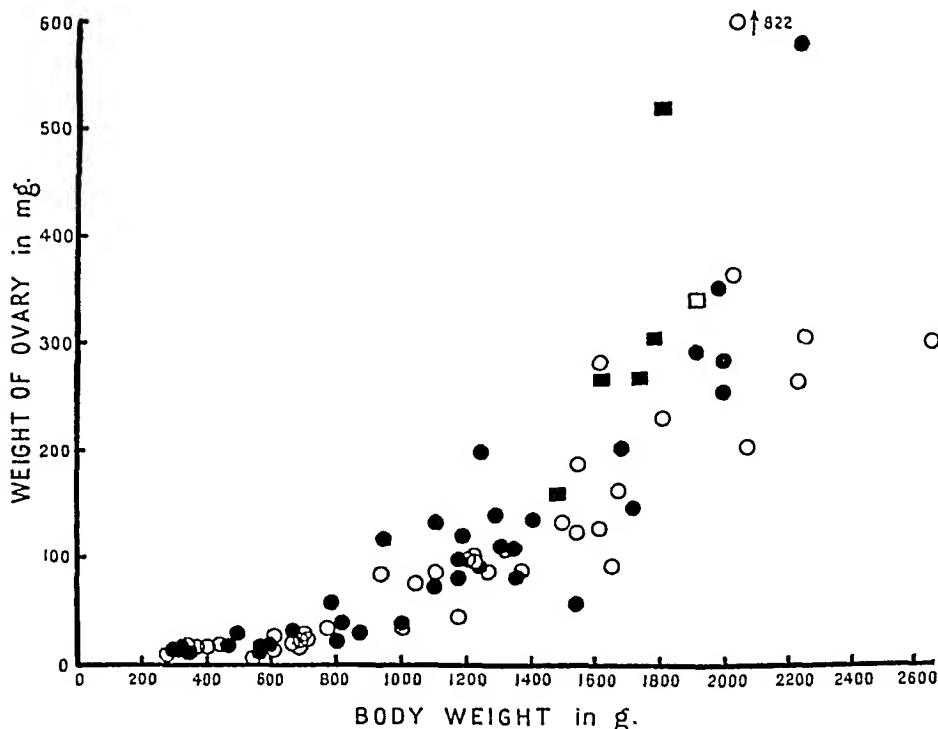
FIG. 1. Ovarian growth in rabbits.
● Dutch rabbits, not ovulated.
■ Dutch rabbits, ovulated.
○ Himalayan rabbits, not ovulated.
□ Himalayan rabbits, ovulated.

this point onwards, with the onset of puberty, growth becomes increasingly rapid, until rabbits with a body-weight of about 2 kg. have ovaries weighing 200–800 mg. There does not appear to be a slowing of the ovarian growth-rate after maturity (these breeds of rabbit do not normally exceed about 2·5 kg. body-weight), nor is there any obvious difference between the ovary-weight curves for Dutch and Himalayan rabbits.

It will be seen from the figure that 5 Dutch and only 1 Himalayan rabbit had ovulated prior to killing. This is in accord with our previous experience, that Dutch rabbits are more liable to ovulate without coitus

than are the Himalayans, although the figures would probably have been lower had all virgin females been strictly isolated from one another. In many instances they were caged in pairs for a period before they were killed.

The curve for rabbits differs from that for rats [Donaldson, 1924] and
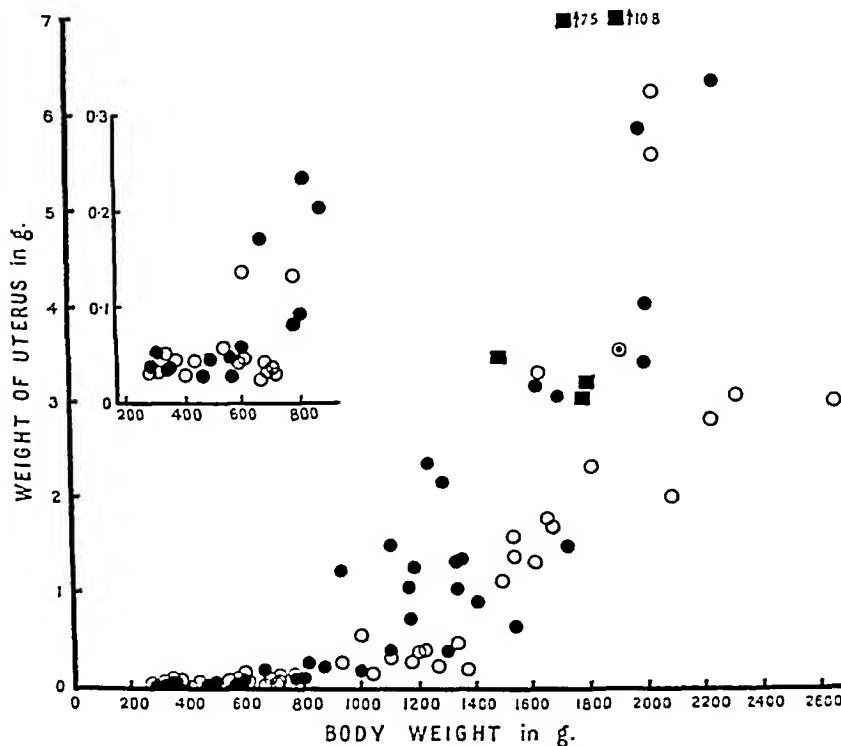
Fig. 2. Uterine growth in rabbits.
● Dutch rabbits, not ovulated.
■ Dutch rabbits, ovulated.
○ Himalayan rabbits, not ovulated.
⊙ Himalayan rabbits, ovulated.

guinea-pigs [Deanesly and Rowlands, 1936] in the absence of flattening at the higher body-weights. In common with that for the guinea-pig, however, the curve shows a more gradual rise at puberty than that for the rat, in which corpora lutea form a large proportion of the ovary. These are not present in the ovary of the virgin rabbit, unless a chance ovulation has occurred within a few weeks of examination.

Uteri

Uterine weights are shown in Fig. 2. The increase in ovarian weight is reflected at the onset of puberty, at a little below a body-weight of 1 kg.,

by an even sharper rise in uterine weight. Below a body-weight of 600 g. the uteri of both breeds are regularly less than 60 mg. Between a body-weight of 600 and 800 g. they may rise to 200 mg., and at about 800 g. a sharp rise occurs in the uterine weights of Dutch rabbits, which may be as much as 2 g. in rabbits of 1·2–1·4 kg. This rise is not so sharp, however, in the Himalayans, until a body-weight of 1·4 kg. is passed. Below 1·4 kg.
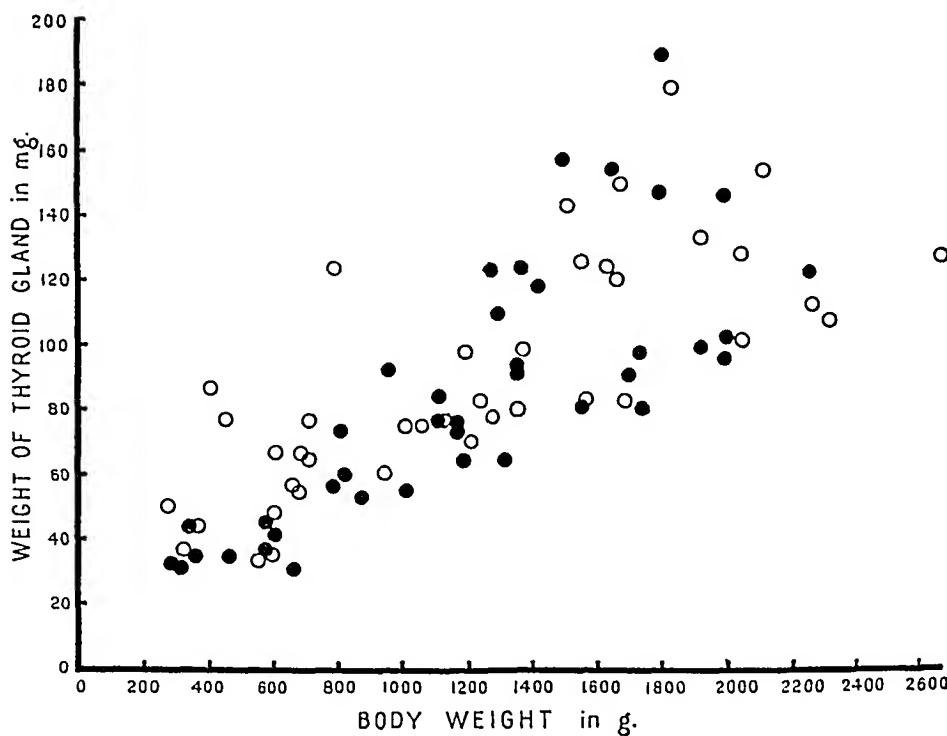
FIG. 3. Growth of the thyroid glands in female rabbits.
● Dutch rabbits.
○ Himalayan rabbits.

body-weight, the uterus of Himalayan rabbits remains less than 600 mg., and is usually about 300 mg. By the time a body-weight of 1·6 kg. is reached, the uterine weight has risen steeply to 1–3 g., and follows in the diagram a line apparently parallel to that for the Dutch rabbits, but shifted towards the heavier end of the body-weight scale by a distance representing about 400 g.

Thyroids

The weight of the thyroids of both breeds appears to bear the same linear relationship to body-weight (Fig. 3). A similar approximately linear relationship was found by Deanesly and Rowlands [1936] for the guinea-pig and by Donaldson [1924] for the rat. The scatter at the higher

ɔody-weights is not so extreme as that found by Deanesly and Rowlands ̣1936] in the guinea-pig.

Adrenals

Figs. 4 and 5 are scatter-diagrams of the weights of the left and right adrenal glands respectively. The left adrenal is usually heavier than the right, but not invariably so. In the rat [Donaldson, 1919] and guinea-pig
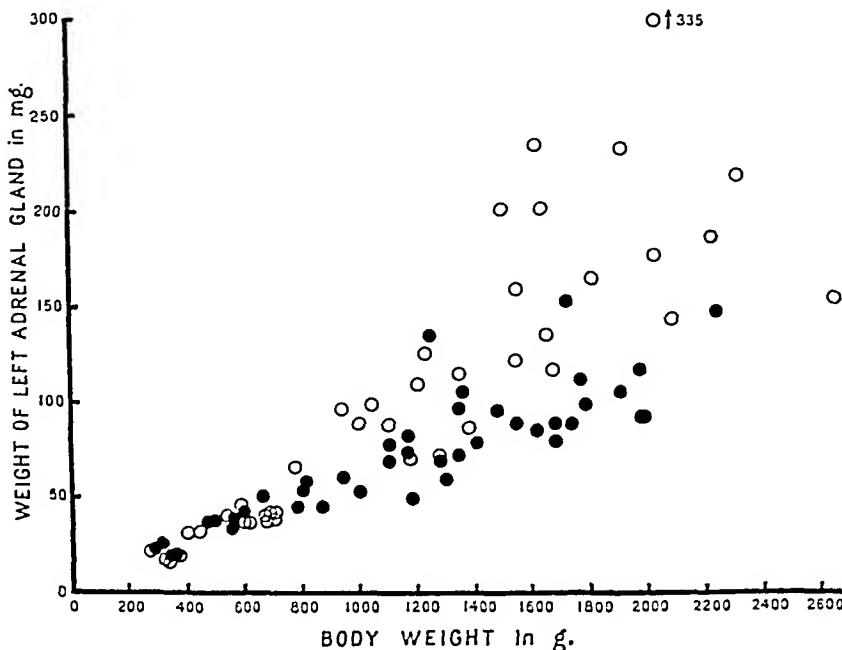
FIG. 4. Growth of the left adrenal gland in female rabbits.
● Dutch rabbits.
○ Himalayan rabbits.

[Deanesly and Rowlands, 1936] it appears that the left adrenal is always the heavier. The relationship of adrenal-weight to body-weight is again approximately linear for both breeds, but the adrenals of Himalayan rabbits increase more rapidly in weight than those of Dutch rabbits. A difference in adrenal-weight between the two breeds is apparent at all body-weights above 1 kg. A further difference between Dutch and Himalayan rabbits, not perhaps obvious from the diagrams, is that the difference in weight between the left and right adrenal is usually greater in the latter. In an extreme case, the left adrenal of a Himalayan rabbit of body-weight 2·02 kg. was 112 mg. heavier than the right adrenal, and a difference of 30–40 mg. is not uncommon. In the Dutch rabbits the greatest difference

found was 21 mg. At the same time, however, it is just as common to find the right adrenal of a Himalayan rabbit heavier than the left (3 cases) as in Dutch rabbits (3 cases).

The scatter of the Himalayan adrenal-weights also appears greater than that for the Dutch rabbits, but this is probably a reflection of the increase in scatter which is usually found as the weight of an organ increases.
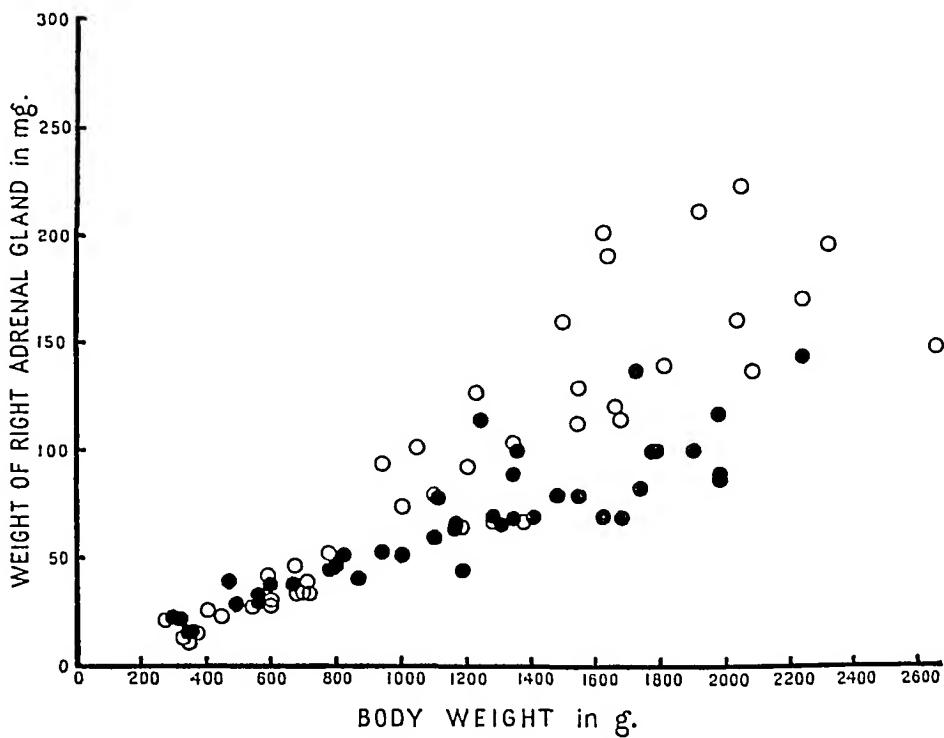
FIG. 5. Growth of the right adrenal gland in female rabbits.
● Dutch rabbits.
○ Himalayan rabbits.

Pituitary gland

The weight of the pituitary gland of some of the rabbits after acetone desiccation is shown in Fig. 6. Its weight is, of course, considerably reduced by this procedure, and probably rendered less reliable. The diagram serves to show, however, that no difference need be suspected between the two breeds of rabbit. The form of the growth curve is not easily seen, as although there appears to be a flattening of the curve from 1 kg. to 1·6 kg. body-weight, this may be a chance phenomenon. On the other hand, this slowing of the growth-rate occurs at about puberty, and may have some significance. Such a slowing was apparently not found by Robb [1928], but Allanson's [1932] diagrams also show it.

DISCUSSION

The main interest of this study, apart from its use for the purposes of controlling experimental work with rabbits, is the difference in some of the growth curves for the two breeds of rabbit.

The later development of the uterus, and the more rapid growth of the adrenal glands in Himalayan rabbits cannot, however, be considered as
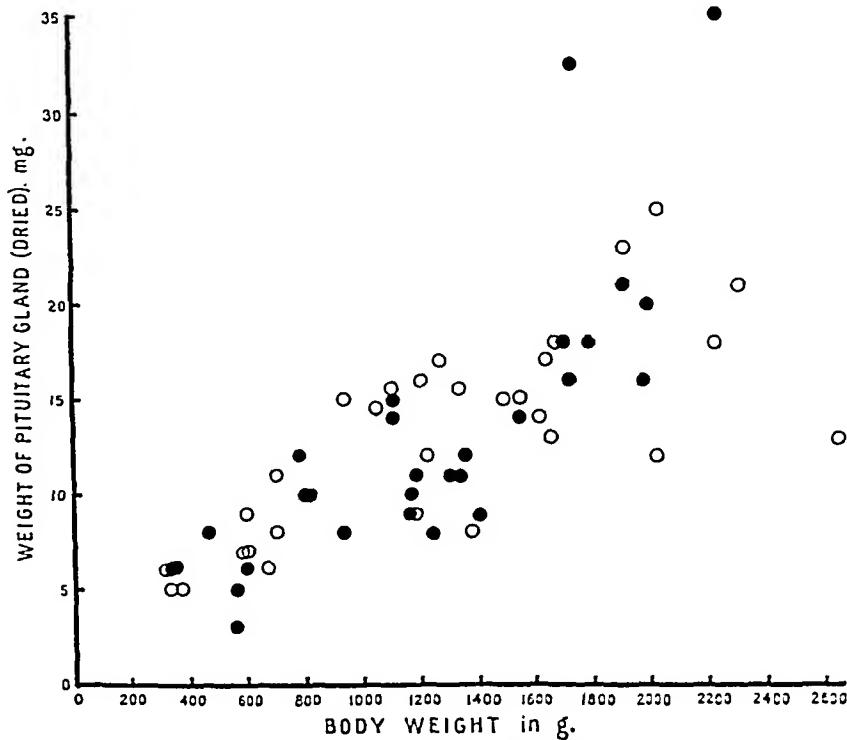
FIG. 6. Growth of the pituitary gland (acetone-dried specimens) in female rabbits.
● Dutch rabbits.
○ Himalayan rabbits.

correlated in any essential fashion with the external differences. The two breeds of rabbit are not crossed, and these differences are in all probability due to a complex of genetic factors which differ in the two strains, and not to environment, since the animals are reared under the same conditions. The older Himalayan rabbits also tend to lay down more fat than the Dutch rabbits, but this cannot be supposed to influence the growth curves for organs in any regular fashion, as the difference in fat deposition is not apparent until a body-weight of more than 1·5 kg. is reached. Moreover, an allowance for this factor would increase the difference

between the adrenal-weights of the two breeds, although not that between uterine weights.

SUMMARY

The growth of the ovaries and uterus, and of the thyroid, adrenal, and pituitary glands has been studied in female Dutch and Himalayan rabbits.

The uterus develops more rapidly in Dutch rabbits than in Himalayan, but the adrenal gland develops more slowly in Dutch rabbits and does not attain the same weight as that in Himalayans. No difference was detected in the other organs.

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THE EFFECT OF PROGESTERONE ON THE GONADOTROPHIC POTENCY OF THE RAT'S PITUITARY

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SEVERAL facts have suggested that the gonadotrophic potency of the pituitary may be diminished by progesterone.

Loeb [1914] noticed that extirpation of the corpora lutea in the guinea-pig was followed by an earlier onset of the next ovulation, and that this occurred even if the animal were in the early stage of pregnancy. More-over, an artificially caused extra-uterine pregnancy, which led to degenera-tion of the corpora lutea, was accompanied by ovulation in spite of the presence of a living embryo within the abdomen. These phenomena might be explained by supposing that the luteal hormone progestin inhibits ovulation by its action on the pituitary.

Papanicolaou [1920], also using the guinea-pig, found that removal of the corpora lutea within 24 hours after oestrus and ovulation accelerated the onset of the next oestrum, the interval between the successive oestra being reduced to 11 days instead of the normal 16–17. Removal of the corpora lutea on the 4th, 8th, or 12th day after oestrus also reduced the interval. From these facts Papanicolaou concluded that the corpora lutea inhibit oestrus. Subsequently [1926] he discovered that a lipoid extract of corpora lutea inhibits oestrus and ovulation in the guinea-pig. Parkes and Bellerby [1927] prepared an extract from cow's corpora lutea which inhibited oestrus in the mouse, Gley [1928] effected a similar inhibition of oestrus in the rat by means of an extract of sow's corpora lutea, and Smith and Engle [1932] were able to prevent menstrual bleeding in the monkey by daily injections of progestin.

Corner [1935] gave daily doses of one rabbit unit of progestin to six rhesus monkeys with the result that no menstrual bleeding occurred so long as the administrations were continued, whereas after cessation of the injections uterine bleeding occurred within 5–8 days. Selye, Brown, and Collip [1936] obtained arrest of oestrus cycles in the rat by daily injections of 4 mg. of progesterone. This treatment, they observed, caused atrophy of the ovary and enlargement of the pituitary gland.

During pregnancy, that is to say in the presence of active corpora lutea,

it has been shown that the gonadotrophic potency of the pituitary is reduced. Bacon [1930] implanted small pieces of the anterior pituitaries of cattle into mice weighing not more than 10 g., and, using the appearance of precocious oestrus as the criterion of gonadotrophic effect, he found that the gonadotrophic content of the cow's pituitary was decidedly reduced during pregnancy. Hill [1932] arrived at a similar conclusion by a different method. He studied female rats which had been joined in parabiosis. When two normal females are united in this way their oestrous cycles are not affected; if, however, one of them is pregnant her fellow goes into a state of continued anoestrus, a condition which suggests a deficiency of gonadotrophic hormone consequent on the pregnancy of the partner. Philipp [1930] tested the pituitaries of 4 men and 12 non-pregnant women, dead from various causes, for gonadotrophic potency. The test animals showed positive responses in every instance. He carried out similar tests with the pituitaries of 10 women who had died during pregnancy or shortly after parturition. Nine of these women had reached the seventh month or a more advanced stage of pregnancy, and in all these cases the test animals gave negative responses. The remaining case was that of a woman who aborted at about the third month, and in this instance a doubtfully positive test was obtained.

Burrows [1939] found that 1 mg. of progesterone given three times a week to young Wistar rats prevented the descent of the testes into the scrotum—an effect which is also produced by androgens and oestrogens, both of which types of hormone are known to check the supply of gonadotrophin by the pituitary. The treatment of undescended testes in boys by injections of gonadotrophin is the result of a not ill-founded belief that gonadotrophin plays an essential part in the descent of the testes into the scrotum.

Despite the suggestiveness of the observations quoted above, the writer was not aware of any direct experimental test of the effect of progesterone on the gonadotrophic potency of the pituitary, and it was to meet this want that the experiment about to be described was undertaken. Since then Herlant [1939] has described experiments on rats to test the matter, and his results show that progesterone given in sufficient doses reduces the gonadotrophin content of the pituitary in the rat.

METHOD

Twelve young adult Wistar rats were used. Six of these were given four daily subcutaneous injections of 5 mg. of progesterone dissolved in 1 c.c. of sesame oil, the other six being given injections of 1 c.c. of sesame oil alone. On the fifth day the rats were killed and their pituitaries were

implanted into female *dba* mice weighing 10–13 g., each mouse receiving one pituitary. The mice were killed four days later and their genital organs were weighed.

RESULTS

In every instance the genital organs were smaller in those mice which had received pituitary implants from the progesterone-treated rats than in the controls. The results are summarized in Table I.

Table I

Source of pituitary implants	Mean weights of organs of recipients expressed in mg. per 10 g. body-weight		
	Uterus	Vagina	Ovaries
Progesterone-treated rats	33·9	39·7	7·6
Control rats	60·5	58·3	10·5

SUMMARY

The results indicate, in so far as the numbers allow any conclusion, that progesterone in the doses employed reduces the gonadotrophin content of the rat's pituitary.

The progesterone used in this experiment was generously supplied by the Ciba Company.

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DISTURBANCE OF GROWTH BY DIETHYL-STILBOESTROL AND OESTRONE

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THE discovery by Dodds, Golberg, Lawson, and Robinson [1938] of a new synthetic oestrogenic substance, diethylstilboestrol, with an oestrogenic potency resembling that of oestrone [Dodds, Lawson, and Noble, 1938; Freud, 1939] has stimulated investigations dealing with the further biological properties of this compound. The action of diethylstilboestrol proves to be similar in many respects to that of oestrone. This applies to its effect on the uterine mucosa [de Fremery and Geerling, 1939], the hypophysis [Noble, 1938], the male genital system [Gaarenstroom, 1939 b], the feathering of cocks and the comb of the capon [Mühlbock, 1939], the sex of the chick embryo [Gaarenstroom, 1939 a], &c.

The synthesis of diethylstilboestrol is comparatively cheap and therefore this compound arouses clinical interest. With regard to its clinical application it was necessary to have exact information about a possible toxic action, since Loeser [1939] and Cobet, Ratschow, and Stehner [1939] recently reported toxic effects following its use in several species of animals, which were not produced by corresponding amounts of oestrone. These effects consisted of vaginal and intestinal bleedings, degenerative changes in liver and kidneys, a disturbance of the general condition, &c. Further, the carcinogenic effect of diethylstilboestrol is claimed to be much greater than that of oestrone [Lacassagne, 1938]. On the other hand, the lethal dose for dogs, following daily administration, proved to be the same for both [Tislowitz, 1939; Arnold, 1939]. The clinical application of diethylstilboestrol seems to be frequently accompanied by toxic symptoms such as nausea, vomiting, abdominal pain, fatigue, and nervousness [Buschbeck and Hausknecht, 1939; Moricard and Saulnier, 1939; Varangot, 1939]. Bishop, Boycott, and Zuckerman [1939], however, observed no notable disturbances arising from its clinical administration. There is some evidence, therefore, to show that diethylstilboestrol possesses a greater activity than oestrone in the production of certain toxic effects. In view of this fact we investigated the extent to which a well-known undesirable effect of oestrone, the growth inhibition following its administration in large doses, is produced by diethylstilboestrol.

As a measure of the rate of growth, the increase in body-weight may be considered. The increase in body-weight, however, is a suitable measure for body-growth only when, during treatment, the constitution of the body is not altered in a qualitative sense. As the occurrence of such an alteration could not be excluded, the increase in length of the bones was

Table I

No. of animals	Daily dose	Duration of treatment Days	Body-weight		
			Before treatment g.	After treatment g.	% increase
8	5 μg. stilboestrol	28	72 (57–84)	103 (92–112)	43
8	5 μg. oestrone	28	72 (60–86)	134 (113–50)	86
8	0·2 c.c. olive oil*	28	74 (66–82)	138 (132–60)	87
5	20 μg. stilboestrol	14	156 (139–71)	150 (134–68)	—4
5	20 μg. oestrone	14	156 (136–98)	191 (176–236)	23
5	0·8 c.c. olive oil	14	150 (143–62)	206 (198–214)	36
6	250 μg. stilboestrol	14	94 (84–103)	97 (86–117)	3
6	250 μg. oestrone	14	96 (86–111)	105 (85–127)	8
8	0·2 c.c. olive oil	14	95 (85–110)	146 (133–81)	54
8	500 μg. stilboestrol	14	99 (86–107)	98 (78–109)	—1
8	500 μg. oestrone	14	84 (70–105)	95 (68–124)	13

* Olive oil, being the solvent of the two oestrogens.

determined as a second criterion. The use of two criteria raises the possibility of conflicting results, the occurrence of which, however, should only confirm the well-known fact that increases in body-weight and bone-growth do not necessarily run parallel.

RESULTS

Inhibition of body-weight increase by diethylstilboestrol.

As may be seen from Table I, the body-growth of immature rats is already remarkably inhibited by the daily administration (by subcutaneous injection) of 5 μg. diethylstilboestrol. After four weeks' treatment the increase in weight was only 43%, that of the control animals being 87%. The same dose of oestrone had no effect whatever on the body-growth, the increase in weight being 86%. When a dosage of 20 μg. was given daily to mature rats, growth was only slightly inhibited by oestrone (increase in weight of oestrone group 23%, controls 36%), but much more by diethylstilboestrol (—4%). After increasing the daily dose to 250–500 μg., body-growth was considerably inhibited by both substances, the effect of oestrone, however, being always less than that of diethylstilboestrol.

Effect of pituitary growth hormone on the inhibition of body-weight increase by oestrone and diethylstilboestrol.

A decrease of the production of growth hormone might account for the inhibition of body-weight increase by oestrogens. We therefore made the following observations to see whether the inhibiting effect of oestrone and diethylstilboestrol on growth was counteracted by the simultaneous administration of growth hormone. The results are given in Table II.

Table II

| | Body-weight | | |
Daily dose	Before treatment g.	After treatment g.	% increase
10 μg. stilboestrol+10 units G.H.*	89 (81–110)	124 (121–8)	39
10 μg. „ +NaOH†	99 (90–114)	138 (120–52)	39
10 μg. oestrone+10 units G.H.	91 (82–106)	142 (132–56)	56
10 μg. „ +NaOH	101 (82–116)	155 (133–91)	54
0·1 c.c. NaOH+0·1 c.c. olive oil	90 (86–92)	156 (139–72)	73
10 μg. stilboestrol+10 units G.H.	104 (95–108)	123 (94–142)	18
10 μg. „ +NaOH	103 (92–106)	110 (101–23)	6
10 μg. oestrone+10 units G.H.	101 (92–106)	136 (122–51)	35
10 μg. „ +NaOH	101 (91–110)	131 (128–34)	30
0·1 c.c. NaOH+0·1 c.c. olive oil	99 (94–104)	136 (127–43)	37
250 μg. stilboestrol+15 units G.H.	90 (82–95)	93 (89–97)	3
250 μg. „	94 (84–103)	97 (86–117)	3
250 μg. oestrone+12 units G.H.	102 (98–105)	122 (98–140)	20
250 μg. „	96 (86–111)	105 (85–127)	8
0·2 c.c. olive oil	98 (87–107)	125 (116–46)	27

In the 10 μg. experiments groups of 4 rats were injected for 21 days, in the 250 μg. experiments groups of 5 rats were injected for 14 days.

* G.H. = growth hormone.
† 0·01 N NaOH, being the solvent of the growth hormone.

Since the daily administration of 10 μg. oestrone had only a slight (if any) inhibiting effect on increase in body-weight, any antagonistic action between this dose and growth hormone was difficult to ascertain. When the dose of oestrone was increased to 250 μg. daily, the growth hormone was found to counteract the growth-inhibiting influence of this dose of oestrone, the increase in weight during 14 days being only 8% after treatment with oestrone and 20% after treatment with both oestrone and growth hormone.

An antagonism between a daily dosage of 10 μg. diethylstilboestrol and growth hormone was not observed in the first experiment but appeared to exist to some extent in the second. In the second experiment, however, the percentage increase in body-weight after combined treatment was abnormally raised by one rapidly growing animal and so probably did not represent the truth. It was also found that the effect of 250 μg.

diethylstilboestrol could not be lessened by the simultaneous administration of growth hormone, and it may be concluded that growth hormone is unable to counteract the inhibition of the increase in body-weight produced by diethylstilboestrol.

Disturbance of bone development produced by diethylstilboestrol and oestrone.

As reported previously [Freud and Levie, 1938; Levie, 1938; Freud, Kroon, and Levie, 1939; Levie, 1939] the point of action of the pituitary growth hormone is shown to be the epiphyseal junction. When, therefore, the action of growth hormone is altered by oestrogens, this should manifest itself by a disturbance in bone development. The growth in length of the bone may be readily controlled by means of skiagrams of the tail of the rat [Freud and Levie, 1938; Levie, 1939], so we used this method to investigate the influence of oestrone and diethylstilboestrol on bone development. This was completed by histological examination of the epiphyseal disks. Groups of rats which weighed from 80 to 90 g. at the beginning of the experiment were treated daily during periods of 14–21 days with 50–1,000 μg. oestrone or diethylstilboestrol. As controls a comparable group of rats received 0·2 c.c. olive oil daily for a similar period. The length of the tail was measured before and after treatment. The data obtained are given in Table III.

As might be expected, oestrone produced a very considerable inhibition of bone-growth, the increase in length of the tail during 14 days, which in the control animals was 13·5–25·0 mm., being reduced to 6·5–9 mm. This effect, resulting from a daily dosage of 250 μg. oestrone, was not increased when the dose was raised to 500 or 1,000 μg., thus proving to be maximal. 50 μg. oestrone daily were found to have no influence on tail-growth. In this connexion it is worth noting that the effect of 200–250 μg. diethylstilboestrol, administered daily, was more pronounced than this maximal effect of oestrone, tail-growth being almost completely arrested. A daily dosage of 10–100 μg. diethylstilboestrol proved to be sufficient to obtain an inhibition of tail-growth equal to that produced by 250–1,000 μg. of oestrone under similar conditions.

Growth in length of other bones was likewise inhibited. The length of the femur, for example, reached only 23·8 mm. when oestrone (500 μg. daily) was administered for 21 days, while the average length in the control animals was 26 mm. (range of 0·1 mm.).

The X-ray pictures of the tail showed that the inhibition of tail-growth was accompanied by the disappearance of a number of epiphyseal apertures. In the control animals an average of 1·6 epiphyseal apertures disappeared during 21 days, while after oestrone treatment 3·2, and after treatment with diethylstilboestrol 8·1 epiphyseal apertures disappeared.

This again demonstrates a significant difference in the relative potencies of oestrone and diethylstilboestrol.

When treatment was discontinued, the tails of the animals of the oestrone group started growing again and the epiphyseal apertures reappeared [Levie, 1938]. The animals which received diethylstilboestrol

Table III

No. of animals	Daily dose µg.	Duration of treatment Days	Average increase in tail-length mm.
Diethylstilboestrol			
6	10	14	10·0
6	20	14	10·6
6	20	13	8·0
6	100	14	9·2
5	200	14	1·6
6	250	14	6·8
6	250	14	1·3
6	500	14	6·8
8	500	19	1·7
Oestrone			
6	50	14	18·2
6	250	14	6·5
6	250	14	4·8
8	500	19	7·0
6	500	14	9·0
6	500	14	10·0
6	500	21	9·0
6	1000	21	9·0
Olive oil	c.c.		
36	0·2	14	19·0
8	0·2	19	19·0
6	0·2	21	38·0
6	0·2	21	38·0

on the contrary showed no increase in tail-length and no reappearance of epiphyseal apertures following the cessation of treatment. Table IV shows the increase in tail-length during a period of 14 days immediately following the last day of injection (see also Plate II, Figs. 6 and 7).

Table IV

No. of animals	Daily treatment	Average increase in tail-length during 14 days immediately following treatment mm.
6	250 µg. stilboestrol	1·2
6	250 µg. ,,	2·8
6	500 µg. ,,	4·4
6	250 µg. oestrone	10·4
6	500 µg. ,,	10·0
6	1000 µg. ,,	14·0
15	0·2 c.c. olive oil	10·5

Toxic action of large doses of diethylstilboestrol and oestrone.

In view of the fact that the damage caused by diethylstilboestrol proved to be in several respects more intense than that brought about by the same amounts of oestrone, it seemed of interest to know if there was any difference in the rate of mortality between the groups when both substances were administered daily in large doses

Table V

Daily dose	Duration of treatment	Average increase in tail-length
	Days	mm.
250 μg. oestrone	14	6·5
250 μg. „ +12 units G.H.*	14	10·0
0·2 c.c. olive oil	14	13·7
250 μg. stilboestrol	14	3·0
250 μg. „ +15 units G.H.	14	3·4
0·2 c.c. olive oil	14	25·0

6 animals in each group.

* G.H. = growth hormone.

Table VI

No. of animals	Weight before treatment	Sex	Daily treatment	Deaths No.	Deaths Time after first injection
	g.				Days
10	20	F	200 μg. stilboestrol	8	8 (2), 9 (4), 11, 16
5	19	F	200 μg. oestrone	2	—
5	20	F	200 μg. oestradiol benzoate	0	—
10	20	F	200 μg. „ „	2	19 (2)
10	22	M	200 μg. stilboestrol	3	11, 15, 17
5	22	M	200 μg. oestrone	0	—
5	23	M	200 μg. oestradiol benzoate	1	18
10	23	M	200 μg. „ „	1	18
5	19	F	50 μg. stilboestrol	0	—
5	19	F	50 μg. oestrone	1	17
5	23	M	50 μg. stilboestrol	0	—
5	22	M	50 μg. oestrone	1	3

The animals were injected for 21 days.

In order to investigate this, daily doses of 50–200 μg. diethylstilboestrol were given to adult male and female mice, other groups of animals receiving the same amounts of oestrone or oestradiol benzoate. A last group was treated with oil only. The rate of mortality occurring during the three weeks treatment is illustrated by the data given in Table VI.

The number of deaths brought about by the daily administration of 200 μg. of the various substances was found to be much larger in the diethylstilboestrol group than in the group to which the other two oestrogens were administered. This fact is especially demonstrated by the death-

rate of the females (8 out of 10 after diethylstilboestrol treatment; 2 out of 10 after oestrone or oestradiol-benzoate treatment). The males in general proved to be more resistant, but the same relative positions regarding toxic properties between diethylstilboestrol and the other two oestrogens existed. Daily treatment with 50 μg. was not sufficient to endanger the life of the animals: the deaths of one male and one female which received oestrone may be regarded as having occurred by chance.

After cessation of treatment the remaining animals were killed. At autopsy the weights of liver, heart, and kidneys proved to be normal. The histological examination of the kidneys revealed nothing worthy of note. The livers of the mice treated with diethylstilboestrol showed an increase of the Kupfer cells, which may be considered a general reaction to intoxication. The livers of the animals treated with oestrone showed the same anomaly to a less degree.

DISCUSSION

The experiments described demonstrate in the first place that the administration of diethylstilboestrol causes a remarkable inhibition of growth. Increase in body-weight as well as in growth of the skeleton was inhibited by relatively small doses (5–20 μg. daily) and totally arrested when the daily dose was increased to 200–50 μg.

The inhibition of growth produced by oestrone was much less pronounced; larger amounts were required to obtain corresponding results and the maximal effect of oestrone (which did not arrest growth) was considerably exceeded by diethylstilboestrol.

A close correlation between the effect of the two oestrogens on body-weight and on bone development exists. It may be concluded, therefore, that the inhibition of body-weight increase is consequent upon the disturbance of bone-growth.

Small doses of oestrone (10–20 μg.) sometimes cause a slight inhibition of body-weight increase without affecting bone-growth. A disturbance in the general condition, causing a decrease of the food intake may account for this.

Since the effect of the larger doses of oestrone on growth inhibition is almost completely counteracted by growth hormone, the effect of oestrone might depend on inhibition of the production of growth hormone by the hypophysis, as already postulated by Zondek [1937]. An antagonistic action *at the epiphyseal junction* between oestrone and growth hormone is, however, a more likely explanation, since the anomalies occurring at the epiphyseal junction after administration of oestrone are not identical with those following hypophysectomy. When the production of growth hormone is alone lacking the epiphyseal junction becomes closed by

formation of a bony plate, which is the typical indication of finished bone-growth. Such a bony plate is never generated by the administration of oestrone.

Neither is a bony plate formed after treatment with diethylstilboestrol, which indicates a certain conformity of action of the two substances. A direct action on the epiphyseal junction may therefore be accepted concerning diethylstilboestrol also. The effect of diethylstilboestrol on the epiphyseal disks, however, is not counteracted by growth hormone and is irreversible; the corresponding effect of oestrone on the other hand is neutralized by growth hormone and is reversible. This supports the supposition that between the effects of oestrone and diethylstilboestrol on the epiphyseal junction not only a quantitative but also a qualitative difference exists.

The higher rate of mortality in mice brought about by diethylstilboestrol, when compared with oestrone, illustrates in another manner the greater damage done by the first substance. The cause of the mortality could not be ascertained, as no significant changes in the organs were observed. The reason may perhaps be found in fatal anaemia, which also occurs in dogs following administration of oestrogens.

From the foregoing it may be concluded that, although diethylstilbo-estrol appears to be in many respects a suitable substitute for oestrone, great care must be exercised in its administration to humans.

SUMMARY

1. The effect of oestrone and diethylstilboestrol in rats and mice was investigated with reference to: (a) weight increase of the body, (b) growth of bone, (c) duration of life.

2. Oestrone inhibited body-weight increase in immature rats with doses of 250 μg. or more (smaller doses only occasionally gave an effect). Growth of bone is likewise inhibited by 250 μg. oestrone or more administered daily, no greater effect being obtained by raising the dose to 1,000 μg. An arrest of growth therefore never occurred. The close correlation between the effect of oestrone on body-weight and on bone-growth favours the conclusion that the inhibition of body-growth is the consequence of the disturbance of bone development. Cessation of the oestrone treatment resulted in the recommencement of bone-growth.

Oestrone does not retard bone-growth by inhibition of the production of growth hormone by the hypophysis, since the effect of oestrone at the epiphyseal junction is not identical with that occurring after hypophys-ectomy. The point of action therefore appears to be the epiphyseal junction.

Daily injection into mice of 200 μg. oestrone killed 2 out of 10 females and 1 out of 10 males within three weeks.

FIG. 1. Growth disk of a proximal vertebra in a rat 9 weeks old, treated daily for 14 days with 0·2 c.c. olive oil. Mallory. × 170.

FIG. 2. Growth disk of a proximal vertebra in a rat 9 weeks old, treated daily during 19 days with 500 μg. diethylstilboestrol. Mallory. × 170.
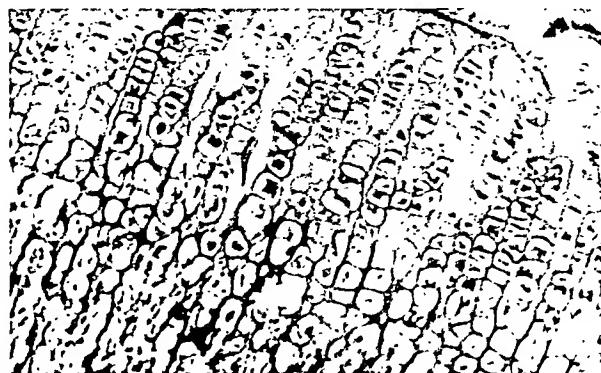
FIG. 3. Proximal growth disk of a tibia in a rat 9 weeks old, treated daily during 14 days with 0·2 c.c. olive oil. Mallory. × 170.

FIG. 4. Proximal growth disk of a tibia in a rat 9 weeks old, treated daily during 14 days with 500 μg. oestrone. Mallory. × 170.

FIG. 5. Proximal growth disk of a tibia in a rat 9 weeks old, treated daily during 14 days with 250 μg. diethylstilboestrol. Mallory. × 170.

FIG. 6. Proximal growth disk of the tibia in a rat 11 weeks old, 14 days after the cessation of the treatment for 14 days with 500 μg. oestrone daily. Mallory. × 170.

FIG. 7. Proximal growth disk of the tibia in a rat 11 weeks old, 14 days after the cessation of the treatment for 14 days with 100 μg. diethylstilboestrol. Mallory. × 170.

FIG. 8. Proximal growth disk of the tibia in a rat 9 weeks old, after simultaneous injection for 14 days with 100 μg. oestrone and 12 U. growth hormone daily. Mallory. × 170.

FIG. 9. Proximal growth disk of the tibia in a rat 9 weeks old, after simultaneous injection for 14 days with daily 100 μg. diethylstilboestrol and 15 U. growth hormone daily. Mallory. × 170.

3. Diethylstilboestrol administered in corresponding amounts inhibited body-growth much more than did oestrone.

Growth of bone is inhibited by a daily dose of as little as 10 μg., growth of bone being arrested by 100 μg. or more daily. No effect of diethylstilboestrol on growth is counteracted by growth hormone. Cessation of treatment with diethylstilboestrol was not followed by a reappearance of bone-growth. The changes occurring at the epiphyseal junction during the administration of diethylstilboestrol were not identical with those following hypophysectomy. So the point of action of diethylstilboestrol, like that of oestrone, seemed to be the epiphyseal junction. Several arguments, however, are in favour of the supposition that between the effects of oestrone and diethylstilboestrol on the epiphyseal disks a difference exists, not only in a quantitative but also in a qualitative sense.

In mice given 200 μg. diethylstilboestrol daily 8 out of 10 females and 3 out of 10 males died within three weeks, the death rate therefore being considerably higher than that resulting from oestrone under the same conditions.

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OBSERVATIONS ON SECONDARY SEXUAL CHARACTERS IN MONKEYS

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TESTOSTERONE is being widely and successfully used in the clinical treatment of hypogonadal conditions in man [e.g. Hamilton, 1937 *a*, *b*; Foss, 1937; Miller, Hubert, and Hamilton, 1938; McCullagh, 1939]. It stimulates the development of the external genitalia and of a masculine distribution of hair; it also heightens the general masculine appearance of the body and face, gives a masculine character to the voice, and induces the mental and behavioural characteristics normal to men. These clinical observations provide more detailed experimental knowledge about the control of secondary sexual characters than is possessed for any other mammal. Very little, for example, is recorded about the endocrine control of secondary sexual characters in sub-human primates. The only observations on the subject of which we know are: (*a*) that the conspicuous cape of hair of the male Hamadryas baboon (*Papio hamadryas*) disappears and the pelage changes to the female type after castration [Antonius, 1930; Zuckerman and Parkes, 1936, 1938, and see below]; and (*b*) that testosterone propionate can lead to increase in weight and to development of sexual-skin coloration and aggressive behaviour in mature castrated male rhesus monkeys [Zuckerman and Parkes, 1938]. The following experiments provide further information which bears on this general question.

HAMADRYAS BABOON (*Papio hamadryas*, 175)

Control observations.

The animal which was the subject of this experiment was a young but mature male Hamadryas baboon which possessed the cape of long annulated silver-grey hair that is the outstanding characteristic of adult males of the species. This cape extends over the nape of the neck, the scalp, and on to the cheeks as long side-whiskers; it ends at the loins. The square-cut bright pink buttock region, in the central part of which are the callosities, is hairless (Plate I, Fig. 1 *a*).

Effects of castration.

The animal was castrated under ether anaesthesia on 19 November 1934. The body-weight at the time was 17·5 kg. Until 8 October 1936 it

received no treatment, and its pelage gradually assumed female character-istics. This change did not become noticeable until the middle of 1935, but from then it proceeded rapidly until only its external genitalia and its longer snout distinguished it at first sight from a mature female of the species (Plate I, Fig. 1 *b*).

The transformation can be best described under the following heads:

Coat. The animal's coat became a brown khaki colour streaked, as in the mature female, with silver. As in the female, the hairs became much more uniform in length, unlike those of the normal male which are much longer on the nape of the neck, the shoulders, and upper part of the back than elsewhere. The hairs of the cape region were mostly either a uniform dull grey-brown or, particularly towards their free ends, annulated in alternate bands of black and brown (Plate I, Fig. 2 *a*). Moreover, the hair of the forearm was greyer than that of the arm, as in the female. The hair of the chest, however, was more silvery, and the cheek-whiskers longer and greyer, than is usual in the female.

Buttocks. The buttock region was less square-cut and smaller than it had been previous to castration. Its lateral and dorso-ventral dimen-sions were 172 and 78 mm. respectively, and the distance between the centre of the anus and a line joining the lower margins of the callosities was 62 mm. Hair covered the rounded margins of the buttock area.

Snout. The snout of the mature male Hamadryas baboon is usually a bright puce or pink. In the castrated animal it was brownish, as in most mature females.

External genitalia. The penis had not noticeably decreased in size and occasionally still became erect, although the frequency of erection became markedly less.

General demeanour. Until the middle of 1935 the animal remained aggressively masculine in its general and sexual behaviour. From then onwards, with the change in its appearance, its attitude became more feminine, and at the beginning of the second phase of the experiment it was no more assertive than a mature female.

The effects of treatment with testosterone.

Between 8 October 1936 and 22 October 1937 the animal was injected once a week with testosterone propionate, 100 mg. being given weekly during the greater part of the experiment. A total of 4·925 g. of the hormone was administered in the whole experimental period. On 19 April 1937 the animal was anaesthetized with ether, measured, and photographed (Plate I, Fig. 1 *c*), and specimens of hair were taken from the regions of the body which had been plucked before castration and before treatment

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was begun. The following changes were observed during and at the end of the experimental period.

Body-weight. During the period of injections the animal's weight increased from 12·66 kg. to 16·19 kg.

Coat. Before the injections were started the hair of the right side of the scalp, of the right shoulder, and of the right side of the back was clipped.

At the end of the first two months of treatment it was noticed that the new hair which grew in the clipped regions was greyer than the 'castrate' hair. At the end of five months the normal cape had been largely restored, and at the end of the experimental period the animal's coat was identical with that of a normal mature male, with the possible exception that there was a wide streak of brownish hair in the mid-dorsal line and a somewhat brownish coloration of the hair of the lower part of the cape and of the hind limbs. The transformation was associated with lengthening of the hair of the cape region, and with alterations in the character of the individual hairs. The grey hairs were much more silvery, and brown had disappeared completely from the fully changed areas, in which the hairs were either a uniform silver or, as over the shoulders, alternately barred black and silver—particularly the distal parts of the hairs (see Plate I, Fig. 2 b). Transformation of the individual hairs in a male direction was complete over the forelimbs, shoulders, and cheeks. It was incomplete on the central part of the scalp, in the mid-dorsal line, and in the lower part of the back, where some brown was still present. It should, however, be noted that both Forbes [1894] and Elliot [1913], who provide the most recent comprehensive reviews of the Primates, regard a certain amount of 'greenish-wash' to be normal to mature male Hamadryas baboons.

Buttocks. About a month after the start of injections the buttocks appeared to be larger, more prominent, and much brighter in colour than they had been previously. On 19 April 1937, when the animal was anaesthetized, the lateral and dorso-ventral diameters of the region had increased from 172 and 78 mm. to 195 and 105 mm., and by the end of the experiment had increased still further to 221 and 112 mm., respectively. The distance between the centre of the anus and the line joining the lower margins of the callosities had not increased. During the latter part of the experiment the buttocks were always a very bright pink. This was presumably due to vascular engorgement, as the skin could be made to blanch on pressure. At autopsy it was also found that the skin of the buttocks was underlaid by a large amount of fibro-fatty tissue.

Snout. Within a fortnight of the start of injections the animal's face began to flush, and it soon assumed the colour normal to mature males.

External genitalia. Erections became more frequent almost immediately

after the start of injections, and the penis, as far as its base, became intensely red. Measurements failed to disclose any significant increase in its length.

General demeanour. As the experiment progressed there was a considerable increase in the animal's vitality, aggressiveness, and sexual interest. This lasted into the latter part of the experiment, when its health began to decline. Its illness took the form of occasional 'fits', and it died without regaining consciousness after a general convulsion. Autopsy failed to reveal any lesion to account for the illness, which had also failed to respond to vitamin B therapy.

<div style="text-align:center">

DRILL (*Mandrillus leucophoeus*, 223)

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Control observations.

The subject of the experiment was an immature drill whose teeth were all deciduous. Together with two other drills of similar dental age it was used for preliminary control observations.

The face of the immature male drill is black, the black mask taking in the brow ridges above and the edge of the lower lip below. Laterally, the black fades somewhat gradually into the bluish-white skin in front of the ears, while below, the black of the lower lip merges gradually with the white of the chin. The muzzle, which is the blackest part of the mask, is more 'pointed' than in maturer animals, and its supero-lateral borders are not especially pronounced or marked by ridges (Plate II, Fig. 3 *a*).

The coat is brownish-black, except on the ventral surface, which is sparsely covered with light hairs. The individual hairs are annulated, black and golden-yellow bands alternating.

The penile sheath, the skin at the base of the penis, and the skin immediately adjacent to the anus and callosities are bright pink, but the scrotum is white, while the skin of the rest of the buttock region is a faint greenish-blue (Plate II, Figs. 3 *b, c*).

Effects of treatment with male hormone.

At the beginning of the period of observations the experimental animal weighed 2·75 kg. Between 31 May 1937 and 5 December 1937 it was injected, usually bi-weekly, with testosterone propionate, the weekly dose starting at 50 mg. and increasing to a maximum of 300 mg. A total of 4·9 g. of the hormone was administered in all. The following changes were observed:

Body-weight. During the six-month experimental period the animal's weight increased from 2·75 kg. to 5·59 kg. At the end of the ensuing year it had further increased only to 6·64 kg., and the animal's first permanent molars had not yet erupted.

Mask. At the end of the first month of injections, when the dose was

only 50 mg. of testosterone propionate weekly, the skin at the diffuse margin between the black of the lower lip and the white of the chin began to redden. This change became more marked in the second month, when the dose was increased to 100 mg. per week, and at the end of the third month the whole mask had altered and had assumed the characteristics seen in the adult male drill. The area of black, instead of fading diffusely at the periphery, was now sharply demarcated, and the black skin had become very shiny. The muzzle had also become more prominent, especially along its supero-lateral borders, due partly to thickening of the skin and partly to growth of the underlying bone.

As the injections were continued, all these changes became more pronounced, and the black area of the mask became even better demarcated by the encroachment of a narrow line of relatively hairless white skin. At a later stage the line of junction of black and white became erythematous. The nasal and supraorbital ridges grew more prominent, and the hair of the chin became lighter in colour. Simultaneously, the white skin behind the ears grew somewhat more conspicuous owing to the recession of hair. In adult males this white patch is very pronounced. Another change which developed was creasing of the skin of the muzzle, the creases being approximately parallel to the line of the mouth. These creases, which could be discerned when the animal's face was at rest, became very obvious when it grimaced (Plate II, Fig. 4a).

Coat. The animal's coat became much thicker, and the hair much longer over the scalp, the shoulders, and the upper part of the back. There was, however, no obvious change in the colour or character of the individual hairs. The ventral surface of the trunk developed a diffuse punctiform flush which was most marked around the nipples.

External genitalia and buttocks. Within a week of the start of injections, the circumanal area of red had extended and become more intense. This change became more marked as the treatment continued, and at one stage the margins of the anus appeared swollen. Later the base of the tail became red and swollen in a roll, and the region between the anus and callosities, in a band about 8 cm. wide, also became very much more prominent and red. Simultaneously the skin surrounding this red area, and extending over the buttocks on to the flanks and lower part of the back, became a brighter blue than the normal immature skin (into which it graded). The zone between the circumanal red area and the peripheral blue area was mauve—due, as could be demonstrated by compressing the skin, to the greater vascularity of that part of the otherwise blue skin. The region between the scrotum and callosities was a greenish-blue (Plate II, Figs. 4b, c. The hair has been removed from the hindquarters in c.)

As these changes developed, the penis became larger and its skin a more

intense red. The red extended in a broad patch over the front of the scrotum and the symphysis pubis and on to the lower abdomen. Three months after the start of injections the scrotum also began to change, first becoming larger and pinker. The colour gradually changed to red, purple, and finally to heliotrope. Around the base of the penis the scrotum remained a brilliant red (Plate II, Fig. 4a). With the growth of the penis, erections became more frequent.

General form. The animal grew much more massive and strong and very stocky. The buttock region became much wider and more square-cut than it had been before.

General demeanour. Although the animal never became too wild to handle, it became much more aggressive, and its mannerisms of threat became those of an adult. It would strut, shake its head, bare its teeth, and crease the skin of its snout in the manner of a fully mature drill.

Variations in colour. It was occasionally observed that in moments of great excitement the red of the animal's chin and anal region became more brilliant. It was also noticed that the red became duller when the dose of hormone was reduced and when the interval between successive injections was prolonged. The red also became less brilliant under ether anaesthesia.

Subsequent observations.

Two days after the last injection the drill was unilaterally castrated. The testis was examined histologically and found to be altogether immature in structure.

The animal was kept under close observation in the eighteen months following the period of injections, and at the end of this period most of the secondary sexual characters which had developed were still unchanged. Thus the facial mask, the bony development of the muzzle, the general body-form, and the penis remained as they were at the end of the injections. The main change which occurred was a diminution of the reddening on the face, trunk, and buttocks. The red at the lateral margins of the mask disappeared completely, as did that on the ventral body-surface. The red of the chin, the anal region, and the penis became less brilliant and less extensive. The heliotrope of the scrotum remained practically unaltered, but the buttock region diminished in size and became more rounded.

During this period, the changes in the animal's behaviour corresponded with those in its secondary sexual characters. It strutted and threatened as if it were a mature male, and its sexual interest continued unabated.

In May 1939, eighteen months after the last injection, a biopsy was made, under ether anaesthesia, of the remaining testis. Histological

examination of the tissue removed showed a normal immature condition. The body-weight at this time was 7·9 kg.

Hanuman Langur (*Presbytis entellus*, 206, 207)

Two immature Hanuman langurs were injected bi-weekly with testosterone propionate for 142 and 157 days, respectively. In both animals the first molar was the only permanent tooth that had erupted by the end of the experiment. During the course of the experiment the body-weight of the first animal (207), which received a total of 0·985 g. of the hormone, increased from 3,300 to 4,590 g., while that of the second (206), which received a total of 1·985 g., increased from 3,740 to 4,390 g. In both animals the testes were altogether immature on histological examination at the end of the experiment.

The only definite external changes which occurred were confined to the genitalia and the buttock area. They were more marked in the animal which received the larger amount of hormone.

In the normal immature langur the penis and scrotum are very small, inconspicuous, and completely black, while the glans penis does not project from the prepuce. By the end of the fourth week of treatment the penis of the injected animal had obviously enlarged, the most conspicuous sign being the projection of the glans from the prepuce, the terminal part of which was becoming pink. As the treatment continued, the penis continued to grow, and the end of the prepuce became marked by a sharply defined pink annular band, about 1·5 cm. wide. This band was continuous with the pink undersurface of the prepuce, and could be made narrower or wider by rolling the prepuce forwards or backwards. At the end of the experiment the scrotum had hardly increased in size, but the testes, which were very small, had fully descended.

The main change in the buttock area was an increase in the dorso-ventral and lateral diameters of the region. The zone of growth appeared to be the dorso-lateral margins of the callosities, and the skin adjacent to the callosities was pinker than that farther away. As a result of this growth, the hinder region of the animal became more massive than is usual in immature langurs.

Comment

The secondary sexual characters of Primates are very varied. In some genera and species, in which there is little sexual dimorphism, they are barely obvious, while in others, for example the mandrill, they are better displayed than perhaps in any non-Primate mammalian species. The development of colours other than shades of brown and grey is especially noteworthy, and it may, perhaps, be correlated with the fact that of

mammals other than man, only monkeys and apes are known with certainty to possess colour-vision.

The three species of monkey described in the present paper may be taken to represent three degrees of elaboration of secondary sexual characters. The langur, like the rhesus monkey described previously [Zuckerman and Parkes, 1938], represents those Primates whose secondary sexual characters, other than gross differences in size, are poorly developed. The baboon represents an intermediate group in which sexual maturity is associated not only with differences in body-form and size, but also with differences in the colour and disposition of the pelage. The drill represents a third and smaller group in which more specialized changes in skin coloration and specialized bone changes are included in the complex of secondary sexual characters. It seems plain, too, that androgenic stimulation induces two main types of change. The first type leads to the assumption of permanent characters such, for example, as the coloration of the mask and scrotum of the drill, and the second to those characters which disappear when androgenic stimulation ceases, e.g. the cape of the male Hamadryas baboon and the red coloration of the buttocks. It is also necessary to bear in mind that the silver-grey cape of the male Hamadryas baboon represents a character which is restricted to males only, whereas the facial mask and maxillary ridges of the male drill, as well as the red coloration of the buttocks, are characteristics which, to some extent at least, are developed in the female of the species and must be considered as potentially ambisexual.

The observations reported in this paper show that characters whose continued existence depend on the continuous activity of male hormone occur in each of the three classes of Primate we have defined. Among these characters are an increased body-weight, an increased prominence of the buttock region, and redness of the skin. The skeletal changes associated with sexual maturity and androgenic stimulation are presumably permanent, and the increase in body-weight and the change in shape of the buttocks cannot, therefore, be ascribed to bone-growth. To what extent they are due to growth of muscle, to the deposition of water, or to the deposition of fat (cf. the fibro-fatty tissue under the skin of the hind quarters of the baboon, p. 432) remains to be determined. The vascular changes would appear to be due to peripheral vasodilatation and to the development of sub-epidermal sinusoidal vessels of the kind normally seen in the sexual skin of female rhesus monkeys [Collings, 1926; Aykroyd and Zuckerman, 1938] and of pig-tailed monkeys [*M. nemestrina*, Maegraith and Zuckerman, 1939]. These changes, while in greater part immediately dependent on either androgenic or oestrogenic stimulation, are to some extent permanent because, as recorded on p. 435, the chin of the experi-

mental drill remained red for more than eighteen months after the cessation of androgenic stimulation. In this connexion we have observed that the brilliant red band which runs down the centre of the muzzle into the red lips of the mandrill (*Mandrillus sphinx*) is due entirely to engorgement of very widely dilated superficial sinusoids, which develop after sexual maturity is reached. The red of these areas can be made to disappear by compression. It may also be observed that, as in the experimental drill reported on in this paper, the various shades from red through mauve to heliotrope blue, which give the hind quarters of the mandrill their brilliant appearance, appear to be due to varying degrees of engorgement of otherwise bluish skin.

The changes induced by androgenic stimulation in the size of the penis appear to be permanent in monkeys. This fact may be correlated with the well-known observation that the penis does not necessarily regress after castration (cf. gelding), and that the size of the penis remains constant in some seasonal mammals. Furthermore, the capacity for erection, which is clearly increased by androgenic stimulation, does not appear, in monkeys, to be altogether due to such stimulation. Finally, while general vitality is increased as a result of the action of androgens, our results also show that great vitality (e.g. that of the young drill) may be exhibited in the absence of obvious androgenic stimulation.

SUMMARY

Castration of the Hamadryas baboon (*Papio hamadryas*) caused loss of the characteristic cape of grey fur, as well as regression of other secondary sexual characters. The condition typical of the normal male was restored by administration of testosterone to the castrate.

The secondary sexual characters typical of adults were induced in an immature drill (*Mandrillus leucophoeus*) and an immature Hanuman langur (*Presbytis entellus*) by the administration of testosterone.

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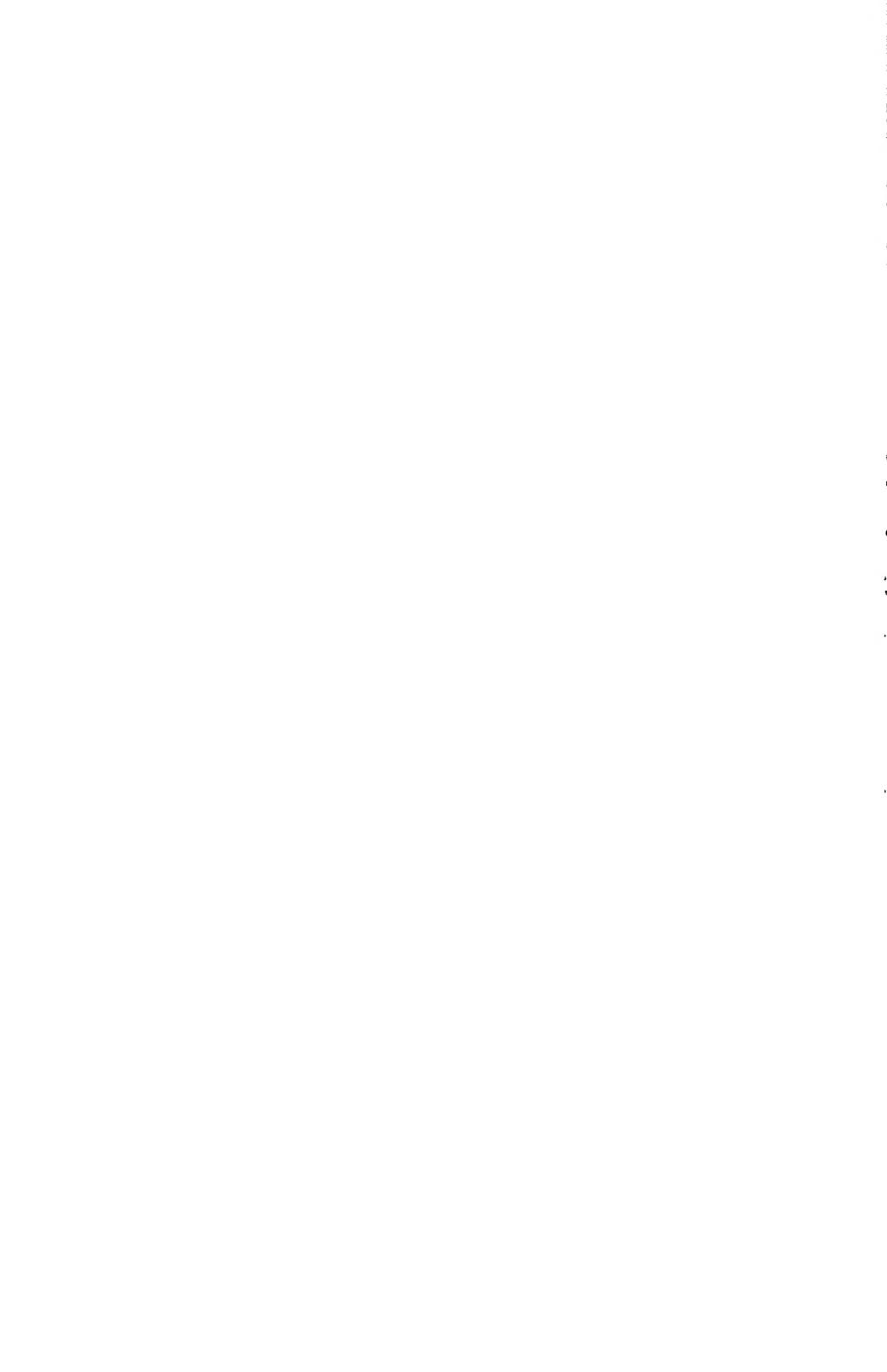
FIG. 3a. Face

FIG. 3c. Circumanal region, showing lack of development of the secondary sexual characters

FIG. 3b. Penis and scrotum

FIG. 4b

FIG. 4a.   Face, penis, and scrotum

FIG. 4c

FIGS. 4b and 4c. Circumanal region and buttocks, showing development caused in the secondary sexual characters

Fig. 1. (a) Baboon in November 1934 before castration, showing luxuriant cape of grey hair.

(b) Same animal in October 1936, two years after castration, showing loss of cape and reduction in buttocks. The pelage is now typically female.

(c) Same animal in April 1937, after six months treatment with testosterone propionate, showing renewed cape and increased buttocks.
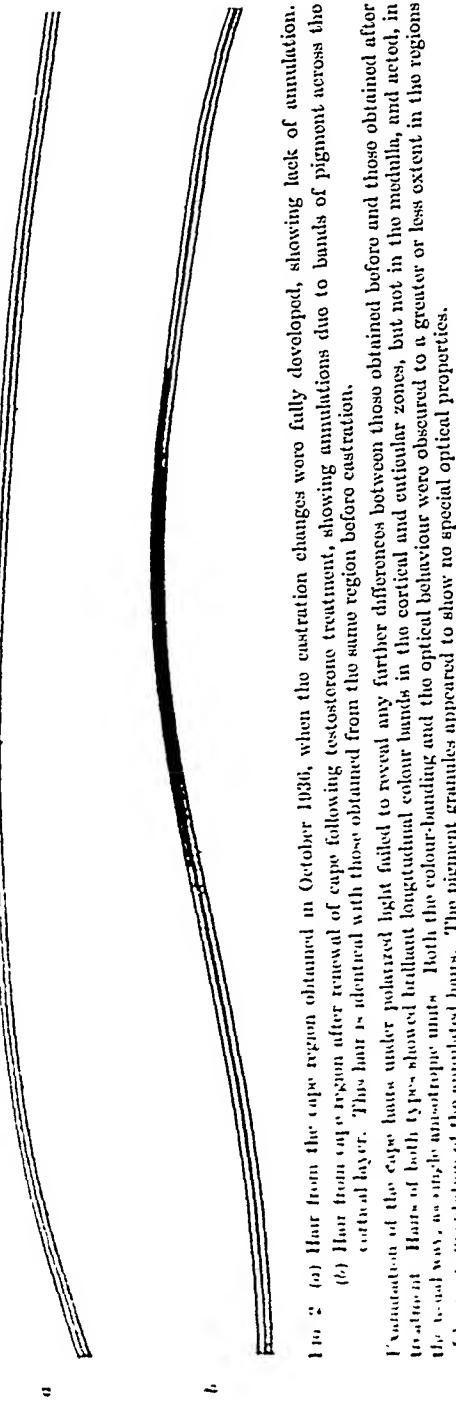
Fig. 2. (a) Hair from the cape region obtained in October 1936, when the castration changes were fully developed, showing lack of annulation.

(b) Hair from cape region after renewal of cape following testosterone treatment, showing annulations due to bands of pigment across the cortical layer. This hair is identical with those obtained from the same region before castration.

Examination of the cape hairs under polarized light failed to reveal any further differences between those obtained before and those obtained after treatment. Hairs of both types showed brilliant longitudinal colour bands in the cortical and cuticular zones, but not in the medulla, and acted, in the usual way, as single anisotropic units. Both the colour-banding and the optical behaviour were obscured to a greater or less extent in the regions of heavy pigmentation of the annulated hairs. The pigment granules appeared to show no special optical properties.

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THE EFFECTS OF DIGESTION BY PROTEOLYTIC ENZYMES ON THE GONADOTROPHIC AND THYROTROPHIC POTENCY OF ANTERIOR PITUITARY EXTRACT

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DURING the recent period of great interest in numerous phases of the physiology of the anterior pituitary, occasional studies of the action of enzymes on hormones present in extracts have been made. With few exceptions, crude enzymes have been used and the assays have been performed in normal rather than hypophysectomized animals. Moreover, there has been no attempt to correlate the extent of digestion with the quantitative or qualitative change in the action of extracts. The investigation reported here attempts to meet these objections. The useful crystalline enzymes of the pancreas and the gastric mucosa were employed. It was thought that pure enzymes attacking specific groups might not only inactivate specific actions of extracts but also facilitate the removal of inert protein. In addition, two crude preparations, a papain and a trypsin, were used. All the assays were performed in immature hypophysectomized rats so that interpretation of the action of an extract could disregard the test animals' own pituitary glands. Lastly, it was found that results may be markedly influenced by the extent of digestion, which was always determined.

The results of earlier investigators are discussed later.

MATERIALS AND METHODS

Preparation of a typical extract of hog pituitary glands.[1] 8·7 kg. of ground fresh hog pituitary glands were extracted with 44 l. of cold 2% NaCl solution in the cold room. After vigorous stirring for about 2 hours, the suspension was allowed to stand in the cold room over night. On the following morning the pH[2] of diluted aliquot samples was adjusted by means of N/70 HCl to the point of maximum precipitation as shown by

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[1] Three different extracts have been used. The description of the method of preparation is typical.

[2] All the determinations of hydrogen-ion concentration were made by means of a glass electrode.

the tube containing the minimum concentration of nitrogen in the supernatant. The calculated amount of N HCl was then added to the large batch; its pH was lowered to within 0·05 unit of the point of maximum precipitation. In various experiments this pH lay in the range 4·2–4·6. The mixture was finally centrifuged and the supernatant was collected in a large crock. The precipitate was washed twice with cold 2% NaCl; about 15 l. were used for each washing. The supernatant and the washings were then combined, and to each litre of fluid were added 660 g. of solid ammonium sulphate. The suspension stood at room temperature over night and was filtered on fluted paper. The precipitate was then dialysed in a refrigerator against distilled water; when free from ammonium sulphate the solution was centrifuged to remove a black sediment which was relatively rich in chromatosome-dispersing hormone. To the supernatant and the washings of the black material was added N NaOH to precipitate some inert protein. The amount of alkali necessary to effect maximum precipitation was previously determined in small aliquot samples in the manner described above. The pH at which the maximum precipitation occurred was approximately 5·10; this inactive precipitate was discarded. After the centrifugation and washing of the precipitate with water, the combined supernatants which contained most of the activity were made up to 33% saturation of ammonium sulphate. The precipitate insoluble at 33% saturation was then centrifuged, after standing at room temperature over night, and washed twice with 33% saturated ammonium sulphate solution. This precipitate was also found to be inactive. The volume of the combined supernatant and washings was measured and from it the equivalence of fresh gland per c.c. solution could be calculated. In order to preserve the solution, we measured aliquots into bottles of convenient size and then added ammonium sulphate to the measured solution to 90% saturation. Just before use the precipitated protein was filtered and dialysed. In such extracts, free from ammonium sulphate, 0·9 mg. of nitrogen was approximately equivalent to 1 g. of fresh gland.

Enzymes used. Trypsin (ox), chymotrypsin (ox), and pepsin (hog), all crystalline preparations, were made available to us through the courtesy of Dr. J. H. Northrop and Dr. M. Kunitz. Dr. M. L. Anson gave us carboxypeptidase which had been crystallized twice but might have contained traces of trypsin. Purified papain was given us by Dr. Hans Lineweaver, who informed us that crystalline papain differed only quantitatively from the purified sample. A sample of commercial trypsin (E. Merck) was also employed and was found to have unusual properties, which are considered later.

Procedure for enzyme digestion. A typical procedure for digesting extract by various enzymes was as follows. A solution of extract (freshly dialysed

so that non-protein N was 5% or lower) in 0·9% saline was diluted so that the final concentration was 0·75 g.-equivalent[1] (0·66 mg. N) per c.c. in 0·25 M buffer. To 20 c.c. aliquots of this solution were added 10 c.c. of a solution of enzyme in 0·05 M buffer of the same pH. The buffer solution without enzyme was used in controls. Crystalline carboxypeptidase, chymotrypsin, and trypsin, and Merck's trypsin were dissolved in phosphate buffer, pH 8·69. Papain and crystalline pepsin were dissolved in acetate buffer, pH 4·57. A few drops of toluene were added as a preservative; digestion was carried out at 37° C. The further procedure and results are outlined in Table I.

Table I.   *Example of digestion procedure (see text)*

| | Enzyme | | Digestion corrected for controls % |
Kind	Final concentration mg./c.c.	Incubation hours	
Alkaline control	0·0	36	—
Carboxypeptidase	0·266	36	12
Chymotrypsin	0·333	18	75
Crystalline trypsin	0·333	18	70
Merck trypsin	0·667	36	65
Acid control	0·0	32	—
Papain	0·667	32	60
Pepsin	0·333	32	70

Samples were taken from time to time to determine the degree of hydrolysis; when two samples taken 8 hours apart showed no significant increase in the percentage of digestion it was assumed that the latter had proceeded to a maximum. In order to be sure that no further hydrolysis could have taken place the following two tests were performed: (a) To 0·5 c.c. of chymotrypsin, trypsin, or Merck-trypsin mixture was added 1·0 c.c. of 0·5% casein in 0·05 M phosphate buffer of pH 7·6. The mixtures were incubated for two hours at 37° C. In all cases casein was digested, showing an excess of enzyme. Similar tests were made on the pepsin, papain, and carboxypeptidase, using as the substrates, gelatin for the first two enzymes and chloroacetyl tyrosine for the last. (b) An increased amount of the appropriate enzyme was added and incubated 12 hours longer without further change in the percentage of protein digested.

Although numerous methods can be used to follow the rate of hydrolysis of protein, we have chosen to determine the decrease of protein precipitable by trichloroacetic acid rather than to determine the increase of amino nitrogen, chiefly because data of the latter method will include cleavage products not only of proteins but also of polypeptides. The procedure used

[1] Higher concentrations of extract were often used in other experiments. Dosage of extracts is expressed throughout in terms of the equivalent weight of fresh gland (g.-equivalent).

was essentially that of Northrop [1932]. Therefore to calculate the percentage digested it is only necessary to determine the decrease of protein nitrogen or

$$\text{percentage digested} = \frac{(PN)_o - (PN)_t}{(PN)_o} \times 100,$$

where $(PN)_o$ is the protein nitrogen found in the acid or alkaline control samples, taken out after a corresponding period of incubation. (The protein nitrogen of the control samples is preferred because there is some digestion of protein in the pituitary extract at the acid or alkaline reactions used.) $(PN)_t$ is the amount of protein nitrogen present at a given time in the extract and is equal to the total protein nitrogen found minus the protein nitrogen present in the enzyme used. This subtraction is negligible unless a large amount of enzyme be used. A further check on the accuracy of the determinations of protein was afforded by determinations of non-protein nitrogen in the trichloroacetic acid supernatant fluids. The experimental error in estimating the amount of digestion (analysis of about 1 mg. total N) is about $\pm 2\%$.

After the digestion was complete, the acid solutions were neutralized. The neutralized as well as the alkaline solutions were made up to volumes corresponding to the dosages required for injection. After proper dilution had been made, the solutions were kept in a frozen state in small vials so that each vial contained just enough material for each injection; this is very important when only partial digestion is desired. To obtain partial digestion of a definite percentage of protein, it was only necessary to incubate the solutions for a shorter period of time than those given in the above table. However, the time for incubation was not the same for all enzymes since digestion took place at different rates. In some experiments in which it was desired to incubate all solutions for the same time and still secure digestion of a certain percentage, we have decreased or increased the amount of enzyme used.

Biological methods. The assays were all performed in immature hypophysectomized male and female rats. A total of 596 completely hypophysectomized rats was used. Operation was performed by a retropharyngeal technique at an age of 21 days and injections were begun 48 hours later. All extracts were injected subcutaneously twice daily. Female animals received injections for 4 days, male animals for 8 days. In either case necropsy was performed 24 hours after the last injection, so that the female rats were 27 days old at death and the male rats were 31 days old. The following fresh tissues were removed and weighed as rapidly as possible on an appropriately sensitive torsion balance: the thyroid (including isthmus), adrenals, spleen, and ovaries and fluid-free uterus (just superior

to cervix), or testes, seminal vesicles together with coagulatory glands (hereafter referred to as seminal vesicles), and anterior lobe of the prostate. The thyroid, seminal vesicles, and prostate were dissected under a binocular microscope. These tissues, with the exception of the spleen in later experiments, were all fixed in Bouin's fluid. In 411 of the animals used, the tissues were sectioned and stained with haematoxylin and eosin. The sella was examined under a binocular microscope. Usually pituitary remnants, if present, could be readily detected; however, if there was any doubt as to the completeness of hypophysectomy, the animal was discarded. Our aim was to test each extract on a group of 5 rats. This aim often could not be realized either because the operation was unexpectedly incomplete in

Table II.   *Weight of fresh organs of rats hypophysectomized at an age of 21 days*

Sex	No. of rats	Age at death	Body-weight at death	Testes	Ant. lobe of prostate	Seminal vesicles[1]	Adrenals	Thyroid	Spleen
		days	g.	mg.	mg.	mg.	mg.	mg.	mg.
M	13	31	42·8±2·2	79·9±2·6	5·19±0·26	5·75±0·18	6·18±0·27	3·92±0·21	106·1±5·7
				Ovaries	Uterus				
F	29	27	35·8±0·7	7·66±0·29	12·12±0·22		7·69±0·30	4·26±0·11	105·6±3·1

In every instance the standard error of the mean is given.
[1] Including coagulatory glands.

some animals or because deaths which were not anticipated occurred. In addition to other controls, groups of operated rats receiving no injections were also used. Part of the data on the weight of the organs from these animals is summarized in Table II, which other investigators may find of interest for purposes of comparison.

The evaluation of results was based on the appearance and weight of the fresh organs and on the histology and measurement by means of a micrometer ocular of certain of the sectioned organs. The histological evaluation was always done without knowledge of the type of treatment. It was found that determination of the height of the thyroid epithelium furnished the best means of detecting a thyrotrophic effect under the conditions of our experiments. Valuable confirmatory data were secured from similar measurements of the uterine epithelium. The diameter of the tubules of the testes varied greatly between animals in a group but confirmed the finding of a significant increase in testicular weight. Similarly, if stimulation was moderate, the diameter of the prostatic alveoli corresponded roughly to prostatic weight. No satisfactory data concerning adrenocortical stimulating effects were secured. In our opinion this was probably owing to the operation of one or several factors, such as low concentration of adrenocortical stimulating principle in the original extracts, lack of sensitivity of the rats following short (4–8 days) periods of

injections, &c. Had apparatus for making frozen sections been available, the cortical lipins would have been examined. We were also unsuccessful in attempts to correlate variations of the weight or histology of the spleen with the nature of the extract injected.

In nearly all the experiments, the significance of differences in mean values as indicated by the probability that the samples were drawn from the same 'population' was determined by the method of Fisher. Values of P equal to 0·05 or less were considered significant in accordance with conventional usage.

<div align="center">RESULTS</div>

<div align="center">*The action of proteolytic enzymes on gonadotrophic hormones*</div>

The effects of various enzymes were found to depend to an important extent on the degree of digestion of the extract. Therefore, extracts were usually subjected to low, moderate, and marked digestion. Frequently, also, various doses were employed in the hope that the degree of alteration or destruction could be estimated. The experiments which are considered in detail are representative of the results obtained.

Enzymes active in an alkaline medium.

Crystalline carboxypeptidase. In terms of the gross weight of the ovaries, there can be no doubt that digestion by carboxypeptidase may be followed by a reduction of the gonadotrophic potency of the extract. Since this enzyme in pure state does not digest any known protein, it is probable that traces of impurities such as trypsin account for both the digestive action of our sample (maximum 12%) and the diminution of gonadotrophic hormone. In various experiments the uterus was sometimes larger and sometimes smaller than that of injected control animals; however, the changes were not statistically significant. In two experiments in which the period of digestion lasted 5 or 30 hours, there was not a significant decrease in the response of the ovaries. The histological appearance of the ovaries of all the animals receiving extract subjected to digestion by carboxypeptidase (4 experiments) indicated that although the potency of the extract might be reduced, the qualitative effects underwent no change (see Plate I, Fig. 11). As in the ovaries of animals receiving undigested extract, follicle-growth, luteinization, and stimulation of the interstitial cells had occurred.

Crystalline chymotrypsin. The gonadotrophic effects of extract after digestion to varying extents by this enzyme were tested (see Figs. 1 and 2). It is clear from observations of both the weight changes and the histology (Plate I, Fig. 12, and Plate VI, Fig. 63) that pronounced digestion of the extract, which in the experiment of Fig. 1 has suffered a reduction of 80%

MERCK'S TRYPSIN = 75%
CRYSTALLINE CARBOXYPEPTIDASE = 10%
" " CHYMOTRYPSIN = 80%
" " TRYPSIN = 75%

WEIGHT OF ORGANS AS PER CENT OF INCUBATED ALKALINE CONTROL (pH=8.69)

OVARIES UTERUS ADRENALS THYROID

←CARBPEPT.
←MERCK'S TRYPS.
CRYST. TRYPS.
CHYMOTRYPS.
←OP. CON.

←M. TRYPS.
←OP. CON.
CARBPEPT.→
MERCK'S TRYPS.→
CHYMOTRYPS.→
CRYST. TRYPS.→
OP. CON.→
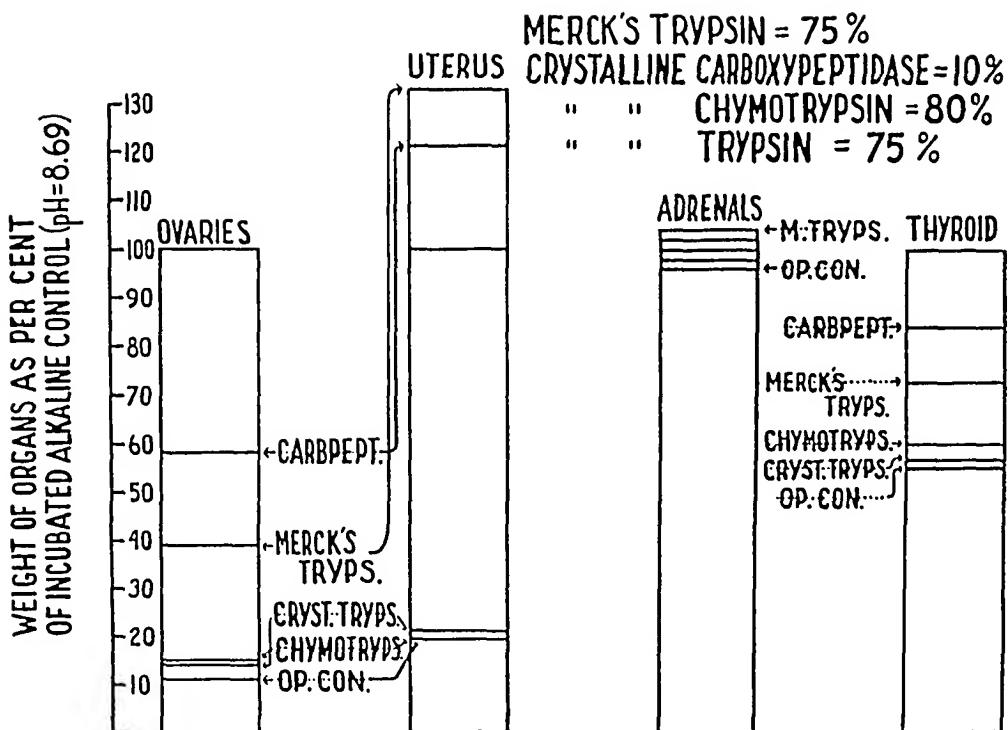
FIG. 1. The effects of enzymes active in an alkaline medium on anterior pituitary extract as shown by the degree of hypertrophy induced in ovaries, uterus, adrenals, and thyroid. The percentage of protein disappearing from each digested extract is indicated by the figure accompanying each enzyme. Four or five hypophysectomized rats in each group. Each rat, except operative controls, received 2 g.-equivalent of fresh hog pituitary.

INCUBATED ALKALINE CONTROL (pH=8.76) MERCK'S TRYPSIN (35%) CRYSTALLINE TRYPSIN (30%) CRYSTALLINE CHYMOTRYPSIN (29%)

AVERAGE WEIGHT OF OVARIES IN MGM.

AVERAGE WEIGHT OF UTERUS IN MGM.

| 4 | 4 | 5 | 3 | 5 | 4 | | 5 | 4 | 4 | | 5 | 4 | 4 | | 5 | 4 | 4 |

OP. CON. 0.25 0.5 1 2 4 | 1 2 4 | 1 2 4 | 1 2 4
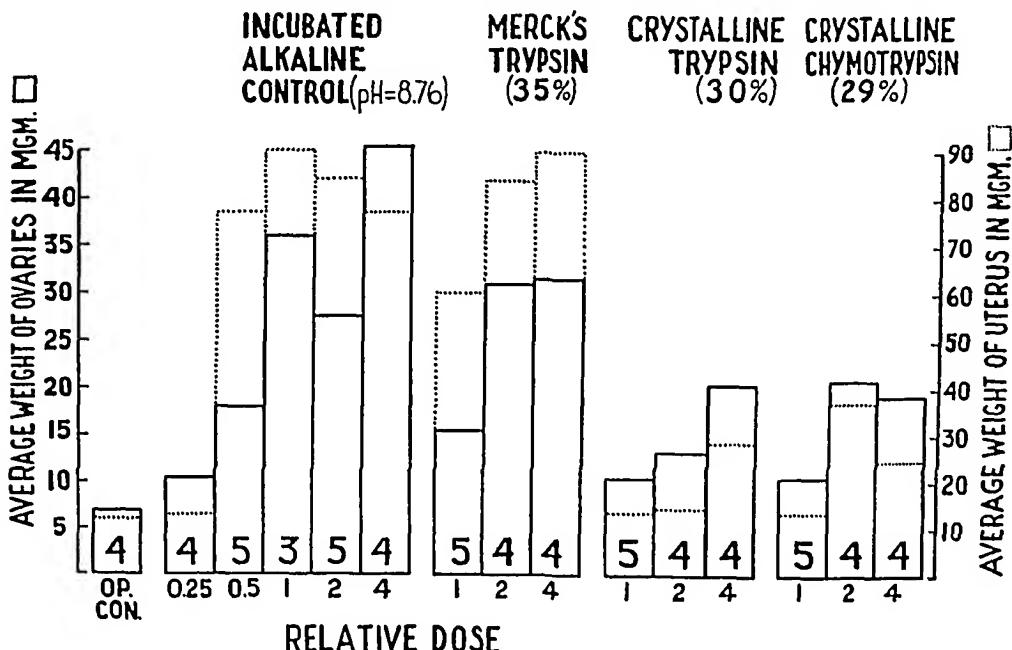
RELATIVE DOSE

FIG. 2. The effects of extracts on the ovaries and uterus after partial digestion (29–35 per cent.) by proteolytic enzymes. The numeral in each rectangle represents the number of rats in each group. A relative dose of 1 is 0·25 g.-equivalent of fresh hog pituitary. Operative controls received no injection.

in the concentration of protein, is accompanied by destruction of most of the gonadotrophic hormone. In some areas slight growth of the follicles could be seen. There was no luteinization and the interstitial cells were as atrophic as in those of untreated hypophysectomized rats. In another experiment, in which 75% of the protein of the extract had been digested, similar results were obtained except that there was some ovarian hypertrophy. Plate I, Fig. 19, and Plate IV, Fig. 45, illustrate the effects of the equivalent of 8 g. of extract (69% of protein digested). There was definite hypertrophy of the ovaries and uterus and a slight stimulation of follicular growth; the interstitial cells were atrophic. .

If digestion was stopped after approximately one-third of the protein had disappeared (29% in Fig. 2; 18, 33, 35,[1] and 45% in other experiments), ovarian hypertrophy varied but was always less than that caused by the incubated control extract. The striking qualitative changes in the ovaries were the persistence of follicle-growth with slight or no luteinization of the thecal cells. The interstitial cells were usually atrophic; however, some stimulation was observed in a few animals. Microphotographs of an ovary of a rat receiving extract partially digested by crystalline chymotrypsin are reproduced in Plate I, Fig. 16, and Plate IV, Fig. 47. Such digested extracts often caused uterine hypertrophy greater than that shown in Fig. 2. However, this hypertrophy was always less than that following the injection of the control extract, although in one instance the change was not significant. The hypertrophy of the uterine epithelium corresponded in general to the change in weight of the whole uterus. Plate VI, Fig. 61, illustrates an exceptionally slight response of the uterus with a definite but slight increase in weight, but probably without any change in the height of the uterine epithelium.

Two different groups of hypophysectomized male rats were each given 4 g.-equivalent of fresh gland after digestion of 65 or 59% of the protein by crystalline chymotrypsin. There occurred much less testicular hypertrophy than in similar rats receiving incubated alkaline control solution, and there was no accompanying hypertrophy of the anterior prostate or seminal vesicles. The histological appearance of the testis may be seen in Plate VII, Fig. 73. In comparison with the testes of hypophysectomized control animals there was definite stimulation of the seminiferous epithelium and the tubules were considerably enlarged.

Since all the histological criteria of stimulation are based on the conditions seen in the testes of untreated hypophysectomized animals, these will be described first. The picture is one of generalized atrophy (Plate VII, Fig. 67). The tubules contain small spermatogonia and numerous Sertoli cells, in all of which the cytoplasmic volume is greatly reduced; the nuclei

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[1] As in the experiment of Fig. 2, three dose levels were used in this experiment.

also appear shrivelled and are crowded near the periphery of the tubule. The flattened and angular appearance of these nuclei is especially characteristic. Mitotic figures are rare, although in occasional tubules they may be seen in greater numbers than is generally appreciated. This is evidence that mitotic waves continue to recur to a limited extent even in the absence of the hypophysis. The interstitial cells show severe cytoplasmic reduction and their pycnotic nuclei stain intensely.

The chymotrypsin digests produced unquestionable swelling of the cytoplasm and nuclei in the epithelial cells (Plate VII, Fig. 73) and mitotic activity was noticeably increased; enlarged spermatogonia, however, were quite rare. The tubule shown in Plate VII, Fig. 73, shows the maximum effect obtained with this digest. As was to be expected from the failure of these extracts to stimulate the ovarian interstitial cells, similar cells of the testes were likewise unaffected.

Incubated alkaline control extracts in comparable dosage (4 g.) produced a marked increase in the diameter of the seminiferous tubules, some of which showed evidence of spermatogonial proliferation. In most tubules, however, widespread cytolysis had occurred leaving a cytoplasmic syncitium (Plate VII, Fig. 68). This was apparently the result of over-stimulation, since these adverse effects were absent in animals receiving the 1 g. dose of this same preparation. Interstitial cell stimulation as shown by increased cytoplasmic volume and granulation of the nuclear chromatin was present in all instances and was roughly proportional to the amount of extract given. Although mitoses are rarely seen in this tissue, even with maximum stimulation, there was undoubtedly an increase in the number of interstitial cells.

Crystalline trypsin. A number of experiments with extracts digested by crystalline trypsin indicated that the changes in activity resembled those following digestion by chymotrypsin. However, there appeared to be a more complete destruction of luteinizing activity. In 4 experiments, 61, 65, 70, and 75% of the protein underwent digestion. In the remaining experiments, the proportions of protein digested were 12, 28, 29, 30, 35, and 48%.[1]

In Fig. 1 it appears that all gonadotrophic activity has been lost as a result of the digestion of 75% of the protein of the extract. Photomicrographs of the ovary and uterus typical of this experiment will be found in Plate I, Fig. 13, and Plate VI, Fig. 64. In all respects the ovaries and uteri resembled those of untreated hypophysectomized rats. In the other experiments (digestion of 61, 65, or 70% of the extract) the only apparent effect was a slight stimulation of follicle-growth even when the dose in-

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[1] In three experiments (28, 30, and 35%) three dose-levels represented by x, $2x$, and $4x$ were investigated.

jected was equivalent to 8 g. gland (Plate I, Fig. 20, and Plate IV, Fig. 46). Although after this large dose there was definite uterine hypertrophy, the height of the epithelium was as low as that in hypophysectomized rats.

The effects of extracts digested to only a moderate extent are illustrated in Fig. 2, Plate I, Fig. 17, Plate IV, Fig. 48, and Plate VI, Fig. 62. Changes in ovarian weight were ordinarily 20–30% of those induced by the control extract, but might be as high as 50% or as low as 10%. Follicle-growth was usually moderately stimulated, but might be slight, especially if the total dose of extract were equivalent to 0·25–0·5 g. fresh gland; the administration of larger doses (1·0–2·0 g.-equivalent) caused definite follicle-growth. Slight thecal luteinization could be observed in three animals (28% digestion). In several others the thecal cells often appeared round rather than spindle-shaped. However, in the majority of the rats there was no evidence either of luteinization or change in the morphology of the thecal cells. The interstitial cells consistently appeared atrophic if the trypsin had hydrolysed 30% of the protein or more.

Despite the apparent stimulation of follicle-growth in many of the rats receiving extract only moderately digested by crystalline trypsin, the growth of the uterus and the hypertrophy of the uterine epithelium were relatively slight, especially in comparison with extracts digested by Merck's trypsin (see the section following this). This was true of extracts digested only to the extent of 12%. Often, although the ovarian changes were clear-cut, no uterine change could be detected. It seems doubtful whether complete follicle-stimulation, as the term is conventionally understood, could be induced after extract had been digested by crystalline trypsin.

In one experiment in hypophysectomized male rats, 4 g.-equivalent of fresh gland after digestion of 65% of the protein by crystalline trypsin were injected into each animal. The results resembled those obtained in male rats given extract digested by crystalline chymotrypsin. Definite but markedly reduced testicular hypertrophy was accompanied by no significant change in the weight of accessory organs. Microscopically these testes could be distinguished from those of untreated animals only by the slightly greater amount of cytoplasm in the spermatogonia (Plate VII, Fig. 74). In comparison with the activity of chymotrypsin digests the stimulating action of trypsin-digested extracts was definitely less pronounced. This is in general agreement with the comparative action of these two preparations on the female gonads. Complete destruction of the principle stimulating the interstitial cells was further confirmed by the atrophic condition of these cells (Plate VII, Fig. 74).

Merck's trypsin. Results of great interest have been obtained by the use of a crude trypsin originally supplied by E. Merck of Darmstadt.[1] This

[1] Thus far our inquiries have supplied us with no information as to the mode of

trypsin was principally employed by Chen and van Dyke [1939] in obtaining extracts which were almost purely follicle-stimulating. We have made a further study of this preparation in respect of its digestive action and of the specificity of the effects of digested extracts. The qualitative and quantitative gonadotrophic effects of extracts in comparison with those of incubated control extract have been determined in groups of hypophysectomized female rats after the following percentages of protein present had disappeared as a result of digestion: 10, 35, 35,[1] 40,[1] 41,[1] 46, 61, 62, 62, 65, and 75.

The effects of digestion to a high level are illustrated in Fig. 1, Plate I, Fig. 14, Plate III, Fig. 40, Plate IV, Figs. 42 and 43, Plate VI, Fig. 65, Plate II, Fig. 22, and Plate V, Fig. 51. Unlike any other enzyme we have employed, digestion to this extent (61–75%) still permits adequate stimulation of the ovaries[2] in which lutein tissue cannot usually be detected. In two of the five experiments small groups of cells perhaps undergoing transformation into lutein cells were observed (Plate IV, Fig. 42). However, such cells are clearly typical and, in our experience, have never appeared following the injection of true luteinizing hormone. In one experiment in which 62% of the protein had been digested, the dose of extract was equivalent to 10 g. of fresh tissue, representing more than 10 times a dose of digested extract which would be clearly effective (ovarian hypertrophy); in the ovaries of these animals no lutein cells could be found and the interstitial tissue was completely atrophic (Plate II, Fig. 22, Plate V, Fig. 51). In all the other animals, except three,[3] receiving an extract in which 65 or 75% of protein had been digested, the interstitial tissue resembled that of hypophysectomized rats receiving no treatment. Another striking feature of the gonadotrophic effects of these extracts was the marked degree of uterine hypertrophy and distension which were always much greater relatively than that of the ovaries. In our opinion this indicates that follicle-growth was physiologically adequate, whereas this was not the case after the administration of extracts digested by crystalline chymotrypsin or crystalline trypsin. Likewise, the increase in the height of the epithelial cells of the uterus in three of the experiments was roughly similar to that in animals treated with control extract (see Plate VI, Fig. 65); in two groups the change was only 25 and 50% of that observed in the injected control groups.

The effects of digestion to lower levels (10–46% of protein in incubated control extract) indicated that some destruction of gonadotrophic activity

source of this preparation or the method by which it was made. The preparation dissolves in water with great ease.

[1] In these experiments, various dose-levels represented by x, $2x$, and $4x$, were employed.

[2] The change in ovarian weight usually corresponded to about 50% of that induced by the incubated control extract.　　　[3] In these three rats only slight stimulation occurred.

might or might not occur depending upon the extent of digestion and the dose used (see Fig. 2, Plate I, Fig. 18, Plate IV, Fig. 49). After digestion had caused the disappearance of 10% of the protein, there was no evidence of destruction of any phase of ovarian or uterine stimulation; moreover, the uterine epithelium was about twice as high as in the treated controls. If digestion was carried farther (35–46%), slight or moderate destruction of gonadotrophic action might occur. Corpora lutea were frequently found, although at low dosage lutein tissue might be absent and yet be found in all injected control animals and in animals receiving larger doses of the digested extract. Substantially similar results were obtained in regard to the stimulation of the interstitial cells. Uterine hypertrophy and the height of the uterine epithelium usually corresponded to those of injected controls. (The results from a relative dose of 1 as illustrated in Fig. 2 are an exception.)

Experiments comparing the action of extract digested by Merck's trypsin with that of incubated control extract were also performed in male animals (see Fig. 5, Plate VII, Figs. 68 and 71). If, as appears logical, digestion by this extract may destroy nearly all luteinizing activity, and if the same substance in extracts is responsible for luteinization in the ovary and interstitial-cell stimulation in the testis, one would expect that the digested extract would cause testicular stimulation almost entirely limited to the germinal epithelium. In two sets of observations (61 and 62% digestion) this belief appeared to receive experimental support. Testicular hypertrophy was found not to be accompanied by any noteworthy change in the interstitial cells, whereas there was marked thickening of the germinal epithelium. Lack of function of the interstitial cells was also indicated by failure of the anterior lobe of the prostate or the seminal vesicles to undergo hypertrophy or histological change.

The seminiferous epithelium showed typical pituitary gametogenic stimulation (Plate VII, Fig. 71). Conspicuous mitotic activity had resulted in marked tubular enlargement. In certain tubules nearly every cell was in a given stage of karyokinesis. Numerous large and apparently mature spermatogonia were present, and meiotic division would undoubtedly have occurred soon had the treatment been continued. The interstitial cells (and accessory sex glands) were not stimulated by a dose (4 g.) sufficient to produce extensive enlargement of the testes; after a dose of 10 g., although the ventral prostate was slightly enlarged, there was no microscopic evidence of interstitial cell stimulation (Plate VIII, Fig. 76).

Enzymes active in an acid medium.

A purified papain and crystalline pepsin were available. Control solutions of extract in the same concentration and at the same pH were incubated

under exactly the same conditions and tested in hypophysectomized male or female rats. Early work demonstrated that the pH cannot be that optimal for pepsin without causing destruction of the hormones in the absence of the enzyme. The lowest pH used was 4·20 with a maximum incubation-period of 48 hours. For most experiments the pH used was about 4·6.
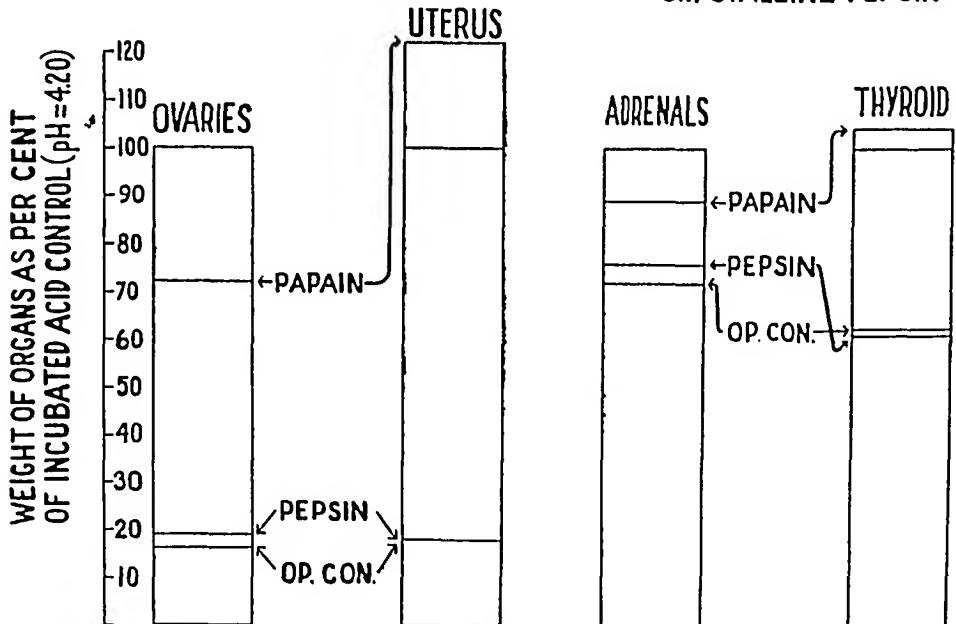
FIG. 3. The effects of extracts, in terms of control extract incubated at the same pH, with and without digestion by papain or crystalline pepsin. Four rats in papain group, five in all other groups. Each rat, except operative controls, received 2 g.-equivalent of fresh hog pituitary.

Papain. The purified papain which was available was used in four experiments in which the proportion of extract-protein digested was estimated to be 6, 31, 60, and 65%. In the experiments in which 6 and 31% of the protein had undergone digestion, there was no evidence of either a qualitative or a quantitative change in the extract. On the other hand, when digestion was carried to higher levels (60, 65%) there was unquestionably a reduction of gonadotrophic action at a dose of 1 g.-equivalent and a probable reduction ($P = 0.05$) at a dose of 2 g.-equivalent as indicated by ovarian weights (see Fig. 3). The uterine weight and the height of the uterine epithelium were not significantly altered in the experiment of Fig. 3. In the other experiment both were significantly reduced. In none of the experiments was there any evidence that specific destruction

of gonadotrophic activity had occurred. Follicular stimulation, luteinization, and stimulation of the interstitial tissue always persisted (see Plate II, Fig. 25).

Crystalline pepsin. The effects of this enzyme were among the most interesting because it appears to destroy all gonadotrophic effects pertaining to the growth and maturation of follicles but leaves intact a stimulating action on the interstitial tissue of the ovaries or testes. However, this is not the result if the percentage of extract-protein digested is low or moderate (10 and 34% in our experiments). Under these conditions, digested extract (34%) may cause less ovarian hypertrophy than the control extract, but the qualitative effects on the ovary are unchanged so that follicle-growth, corpus-luteum formation, and stimulation of interstitial tissue occur (see Plate II, Fig. 28). Therefore the greater part of our attention was given to the determination of the action of extracts digested markedly or to the maximum feasible extent. The percentages of extract-protein digested are represented by the following figures: 58,[1] 66, 69, 70, 75, and 80.[1]

Provided that the greater part of the protein (58–80%) had disappeared as a result of peptic digestion, extracts rarely caused any ovarian hypertrophy. In only one of seven groups of experiments was there a slight ovarian hypertrophy amounting to 15% of the change induced by the control extract incubated in a solution of the same pH. The total dose was equivalent to 2·0 g. of fresh gland in this experiment; in the other six experiments it amounted to 0·5, 0·88, 1·0, 1·0, 2·0, and 2·0 g. Doses representing 6 and 10 g. were followed by ovarian hypertrophy amounting to 30 and 55% of the change caused by 0·5 g. and 10 g. respectively of incubated control extract. Follicle-growth and corpus luteum formation were present only after the largest dose; following the 6 g. dose, a few small antra-containing follicles showing considerable swelling of the thecal cells were present. Slight follicular development without luteinization was observed in two groups on doses of 2 g. only; in one of these groups the change in ovarian weight was 15% of that caused by the control extract. Definite uterine hypertrophy accompanied by enlargement of the cells of the epithelium occurred only in the two groups receiving 6 and 10 g. of extract.

One invariable change, a stimulation of the interstitial cells of the ovary, was observed in all the rats irrespective of the period of digestion or the equivalent dose of extract, the smallest dose of which was 0·5 g. The weight changes are illustrated in Figs. 3 and 4, the histological changes in Plate II, Figs. 26 and 30, Plate III, Figs. 36 and 41, Plate V, Figs. 56 and 57.

[1] These digests were investigated at three or two dose levels.

Apart from the primordial follicles, the ovaries appeared to consist of hypertrophied interstitial cells such as those illustrated in Plate III, Fig. 41, and Plate V, Figs. 56 and 57. In contrast, the normal immature rat's ovary is made up of connective tissue, islands of interstitial cells, and follicles in various stages of development. Various interpretations have been placed on the significance of the ovarian interstitial tissue. Discussion
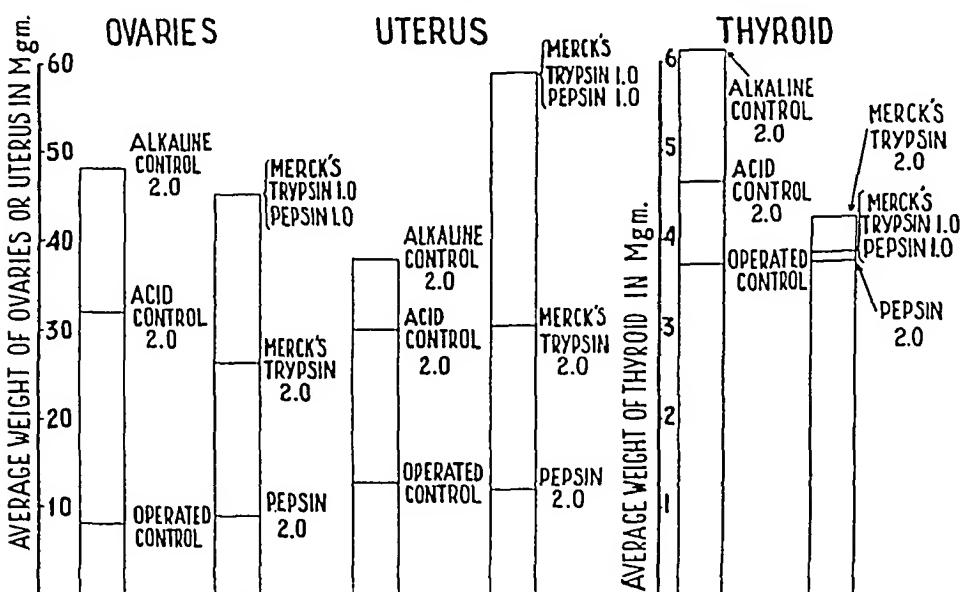
FIG. 4. Weights of various organs of female hypophysectomized rats after administration of control extracts or of extracts digested by Merck's trypsin or by crystalline pepsin either alone or in combination. Operated controls not treated. Four or five rats in each group. Total doses in g.-equivalent of fresh hog pituitary indicated by numerals.

of these views appears later. A working hypothesis for which no originality is claimed is as follows: stimulation of the interstitial cells of the ovary will be the only apparent change induced in hypophysectomized rats by luteinizing hormone which is identical with interstitial-cell stimulating hormone; if follicular growth be adequately stimulated, luteinizing hormone brings about luteinization of the theca (and granulosa) as well as stimulation of the interstitial cells; the interstitial cells of the testis are likewise stimulated by luteinizing hormone so that androgen is liberated with stimulation of male accessory organs and, ultimately, maintenance of the germinal epithelium even in the absence of the pituitary or of administration of follicle-stimulating (gametogenic) hormone. Tests of the third part of the hypothesis were made in two groups of experiments on hypophysectomized immature male rats. Weight-changes of one group of experiments are illustrated in Fig. 5, histological changes in the testes

in Plate VII, Fig. 70. The experiments indicated beyond question that extract adequately digested by pepsin induced testicular hypertrophy and hypertrophy of the anterior lobe of the prostate. We were not able to show that significant hypertrophy of the seminal vesicles, which we have consistently found are difficult to stimulate grossly under the conditions of our experiments, was produced.

PER CENT PROTEIN DIGESTED BY MERCK'S TRYPSIN = 61
"   "   "   "   "   " CRYSTALLINE PEPSIN = 66
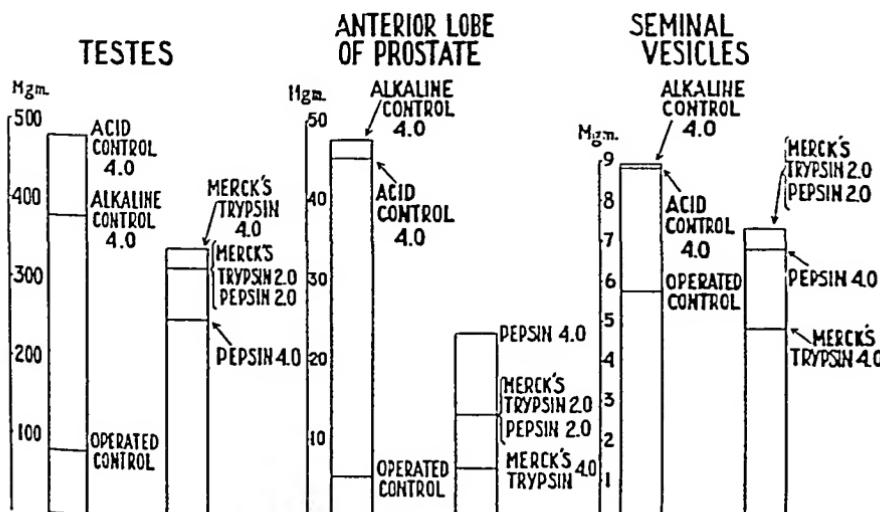
Fig. 5. An experiment like that of Fig. 4 but performed in male hypophysectomized rats. Four rats in 'Merck's trypsin' group, five in all other groups.

Extracts digested by pepsin have consistently produced hypertrophy of the seminiferous tubules and of the interstitial cells (Plate VII, Fig. 70). Although the extent of tubular enlargement in comparison with that produced by Merck-trypsin digests was always less at any given dosage, the degree of spermatogonial proliferation was similar. The interstitial cells were not uniformly stimulated in any one animal by the 4 g. dosage of peptic digests, but the stimulation was unquestionable (Plate VII, Fig. 70) in the major portion of the testes in all instances. With a higher dose (10 g.) the stimulation was somewhat more marked (Plate VIII, Fig. 77). The weights of the accessory glands and the extent of interstitial-cell hypertrophy induced by peptic digest as compared with non-digested control extract (Plate VII, Fig. 69) indicated that the interstitial-cell stimulating activity of the original extract was definitely reduced by peptic digestion. The maximum height of the epithelial cells of the seminal vesicles of rats receiving undigested extract was only slightly greater than

that of untreated controls. In the anterior prostate, the maximum increase of epithelial height in terms of specimens from untreated controls was greatly increased both by peptic digests and by undigested extract; however, the extreme variation found throughout any single prostate made any judgement of the degree of stimulation unreliable. Tall columnar epithelium is present in the alveoli in the early stage of secretion, but, as semen accumulates and causes alveolar distension, the epithelium becomes secretorially 'exhausted' and resembles a flat membrane. The mean diameter of the 20 largest alveoli in a given section was measured. In untreated controls, the alveoli were small bud-like structures (av. 59 micra) whereas, after the injection of control (av. 207 micra) or pepsin-digested extracts (av. 160 micra), they were greatly enlarged owing, ultimately, to the stimulation of the testicular interstitial cells. Under these conditions, especially when prostatic hypertrophy was moderate, the diameters of the largest alveoli were roughly proportional to prostatic weights.

The hypothesis outlined (p. 454) has likewise been tested by means of experiments in which extracts digested by crystalline pepsin or by Merck's trypsin, alone or in combination, have been administered to hypophysectomized immature female or male rats. (All injections were made subcutaneously; when both digests were administered to one animal, they were mixed together before injection.) Appropriate undigested control solutions of the extracts were also injected. The weight changes observed are given in the diagrams of Figs. 4 and 5. The typical histological changes are illustrated in Plate III, Figs. 33–8 (females), and Plate VII, Figs. 67–72 (males). In such a combination experiment presumably the Merck-trypsin digests would furnish follicle-stimulating (gametogenic) hormone almost exclusively whereas peptic digests would furnish luteinizing (interstitial-cell stimulating) hormone contaminated only by insignificant amounts of follicle-stimulating hormone. This belief is supported by the control experiments in which either extract alone was injected. To increase the significance of the results the total equivalent doses of fresh gland administered were the same in all groups (Fig. 4). In the female rats the expected changes occurred. The ovarian weight after the combined extract was administered was greater, but not significantly greater ($P = 0.1$), than after the administration of the tryptic digest alone. Corpora lutea were formed in the ovaries of animals receiving the combined treatment but were absent from the ovaries of rats receiving either component in twice the dose. The average uterine weight was significantly greater after the combined extracts than after the extract digested by Merck's trypsin ($P = <0.001$) or after the peptic digest. The uterine epithelium was highest in the rats receiving combined treatment, and in this respect was equalled by no other group including groups receiving undigested control

extracts. The experiments carried out on male animals have less signifi-
cance because it is probable that, if hypophysectomized rats were used, the
injection of androgen or the stimulation of interstitial-cell secretion may
adequately maintain the germinal epithelium. Hence, in male animals,
the results largely confirm those expected from experiments in which
either digested extract was injected separately (Fig. 5 and Plate VII,
Figs. 67–72). It is evident that peptic digestion has destroyed part of the
hormone stimulating the interstitial cells. For example, after the injection
of the peptic digest the weight of the anterior prostate is significantly less
($P = 0·01$) than after the injection of the acid control extract. As was
mentioned previously, the seminal vesicles were not readily stimulated
in our experiments if judgement be based on change in weight. However,
in comparison with the action of extracts digested by Merck's trypsin,
peptic digest or combined treatment caused a significantly greater hyper-
trophy ($P = 0·01$). Histologically the testes of rats treated with Merck-
trypsin digest (2 g.) combined with peptic digest (2 g.) showed excellent
spermatogonial proliferation (Plate VII, Fig. 72). The interstitial stimu-
lation was less marked than that produced by twice this dosage of peptic
digest alone. Plate VII, Fig. 72, shows an area of maximum interstitial-
cell stimulation produced by the combined digests.

The action of proteolytic enzymes on thyrotrophic hormone

Our data also yield considerable information on the action of the various
enzymes on thyrotrophic hormone. Thyrotrophic effects in hyphophys-
ectomized rats may be judged by an increase in thyroid weight or by
histological changes in the thyroid or by both. In our experiments much
more reliable data were obtained by placing chief reliance on the height
of the thyroid epithelium which could be measured and compared with
corresponding measurements of glands from treated or untreated control
animals.[1] In untreated hypophysectomized rats, the average height of
the thyroid epithelium was 3·8 micra in females and 3·9 micra in males.
Extract might cause an elevation of height to 9 micra without significant
change in thyroid weight. The maximum height observed, and this was
usually associated with the maximum hypertrophy of the thyroid, was
14·4 micra. Ordinarily the average of 30 measurements in each group of
rats was a sufficient basis for conclusions.

Crystalline carboxypeptidase. In three of the four experiments the change
in thyroid weight caused by digested extract was 60–80% of that caused by
the control extract. However, the difference in effect was not statistically
significant in any experiment. The height of the thyroid epithelium was
not less in the glands of animals receiving digested extract in comparison

[1] The glands were fixed in Bouin's fluid. Hematoxylin and eosin were used as stains.

with those receiving control extract. It is necessary to conclude that an effect of crystalline carboxypeptidase on the thyrotrophic activity of hog pituitary extract could not be demonstrated.

Crystalline chymotrypsin. Fifteen groups of hypophysectomized rats (13 female and 2 male), for which there were appropriate control groups which were either untreated or had received similar amounts of undigested extract, received extract which had been digested by chymotrypsin. The
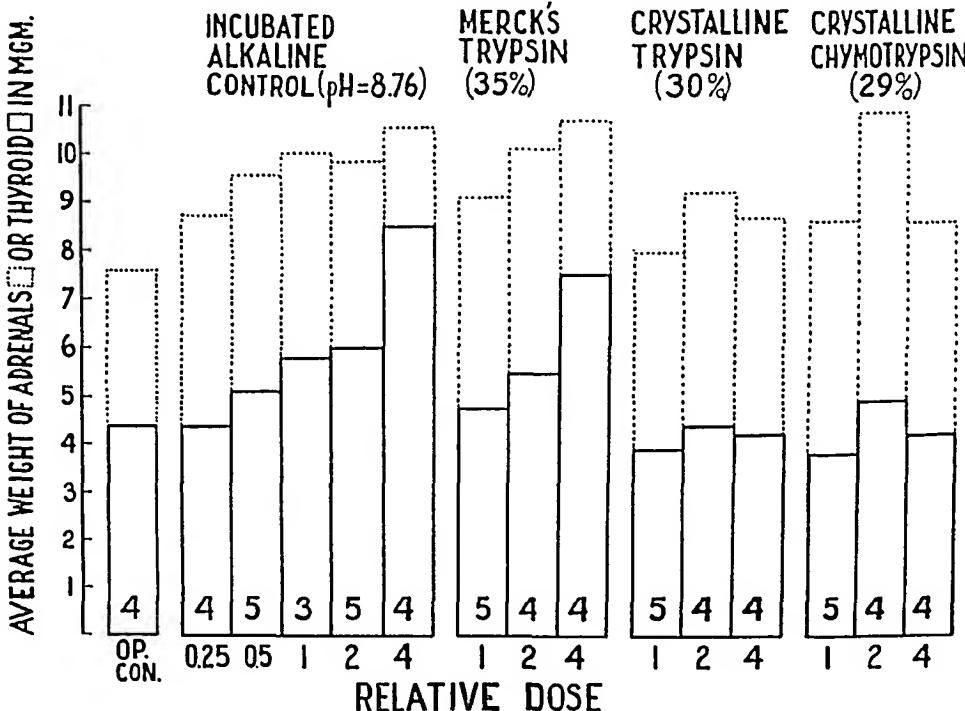
FIG. 6. The weights of adrenals and thyroid of the rats of the experiment of Fig. 2.

percentage of protein digested ranged from 18 to 80. The g.-equivalent of fresh gland administered varied from 0·25 to 8·0. In not a single group was there any manifestation of a thyrotrophic action as indicated by a significant change either in thyroid weight or in the height of the follicular epithelium. On the other hand, in every control group, including those on the lowest doses of undigested extract, the height of the thyroid epithelium was unquestionably greater than that of untreated controls. On the lowest doses only (0·125–0·5 g.-equivalent), the control extract might not cause a significant increase in the weight of the thyroid. Figs. 1 and 6 and Plate IX, Figs. 87 and 89, illustrate our findings. There appears to be little doubt that the thyrotrophic hormone in our extract was rapidly inactivated by chymotrypsin.

Crystalline trypsin. The results of the experiments with crystalline trypsin like those with crystalline chymotrypsin are remarkably uniform

and indicate that thyrotrophic hormone also becomes inactive if incubated in the presence of crystalline trypsin. Sixteen of the 17 groups of rats used were female. The degree of digestion as indicated by the disappearance of protein varied from 12 to 75%. The incubated control extract, even in doses equivalent to 0·125 g. of fresh gland, was always effective with the qualifications mentioned in the preceding section. There was no evidence of any thyroid stimulation in rats receiving digested extract. Data representative of the rest of the experiments are illustrated by Figs. 1 and 6 and Plate IX, Fig. 88. It is possible that a slight thyrotrophic effect is shown in Plate IX, Fig. 90, although a similar appearance of the thyroid epithelium was not found in the greater part of the sections or in those of the thyroids of the other rats of this group. It is, therefore, probable that thyrotrophic hormone is quickly destroyed by crystalline trypsin under proper conditions.

Merck's trypsin. The experiments in which extract digested by Merck's trypsin was injected required the use of 17 groups of female hyphophysectomized rats and three groups of males. The amount of protein removed by digestion varied between the limits of 10 and 75%. Eight of the 20 groups received extract in which more than 60% of the protein had disappeared. The range of dosage was 0·25 to 10 g.-equivalents. Undigested control extracts were always active in terms of the description already given.

Our data indicate that the particular sample of Merck's trypsin used does not ordinarily inactivate the thyrotrophic hormone of our extracts. If all the experiments be considered together, the mean increase of thyroid weight induced by digested extract, expressed as percentage of change caused by the control extract, was 63%; the corresponding figure for the increase in height of the epithelium of the follicles was 77%. No thyrotrophic action could be demonstrated in two groups of rats, one male and one female, receiving 2 or 4 g.-equivalents of extract from which 62% of protein had been removed by digestion (see Fig. 4). In the other six comparable experiments (percentage of digestion, 61–75%; dosage, 1, 2, 2, 4, 10, 10 g.-equivalent) a thyrotrophic effect was always demonstrated by an increase in the height of the follicular epithelium, although a significant increase in thyroid weight was absent in two of the groups in which, however, there was evidence of maximum stimulation with disappearance of nearly all the colloid. We believe that Merck's trypsin may partially inactivate thyrotrophic hormone but that we have only a rough idea of the extent of this inactivation. On the other hand, thyrotrophic hormone may lose none of its activity even after nearly half of the protein present in an extract has undergone digestion. Part of the data under discussion is illustrated in the bar diagrams of Figs. 1, 4, and 6. A microphotograph illustrating microscopical changes is reproduced in Plate IX, Fig. 83.

Papain. The incubated control extract for both papain and pepsin was, of course, kept at an acid pH. The dosage of such control extracts in conformity with the digested extracts was usually 1–2 g.-equivalent but sometimes was as low as 0·5 g. or as high as 10 g. In every group, which included groups of male rats, the control extract caused hypertrophy of the thyroid together with an increase of the height of the epithelium.

The effect of digestion by papain on the thyrotrophic action of extracts is illustrated in Fig. 3. In the four experiments performed (digestion: 6, 31, 60, and 65%; dosage: 2, 2, 1, and 2 g.-equivalent) there was no evidence of destruction of thyrotrophic hormone. The thyrotrophic action of extracts in respect of hypertrophy of the thyroid or of the epithelium of the gland resembled that induced by control extracts.

Crystalline pepsin. Digestion by this enzyme was found to be one of the most effective means of abolishing the thyrotrophic action of an extract. The 14 groups of rats used consisted of 10 female groups and 4 male groups. Ten to 80% of the protein of the extracts was removed by digestion (10, 34, 58, 66, 69, 70, 75, and 80%). The dosages used ranged from 0·5 to 10 g.-equivalent (0·5, 1, 1, 2, 2, 2, 2, 2, 2, 4, 4, 6, 10, and 10 g.-equivalent); 12 of the 14 groups received extract digested to the extent of 58% or higher. The following failures completely to abolish a thyrotrophic action by extract occurred: thyrotrophic effects were found in the group receiving a '10%' digest in a dose of 2 g.-equivalent; a stimulation of thyroid epithelium only occurred in some animals receiving 2 g.-equivalent of the extract in which 66% of the protein had undergone digestion; finally, in one animal each of the groups of females receiving 6 or 10 g.-equivalent of extract, there was definite enlargement of the epithelial cells without change in thyroid weight. In all the other animals receiving pepsin-digested extract there was no evidence of any thyroid stimulation. This fact is of interest because luteinizing (interstitial-cell stimulating) hormone was not strikingly affected by peptic digestion. In fact, a high dose of an extract (6 g.-equivalent after digestion of 80% of the protein or 10 g.-equivalent after digestion of 58% of the protein) might reveal some follicle-stimulating action in addition to interstitial-cell stimulation without, however, any evidence of thyroid stimulation. Such doses represented 12–20 times the dose of digested extract causing definite interstitial-cell stimulation. (See Plate V, Fig. 57, and Plate IX, Figs. 85 and 86.) Other experiments are illustrated in Figs. 3 and 4, and Plate IX, Fig. 84.

The effects of extracts on the adrenal glands

When our first experiments were planned, we expected to gain considerable information on the behaviour of adrenal-cortical stimulating

hormone, after the hormone, which presumably was present in our extracts, had been subjected to digestion by various enzymes. However, a careful study of the weights of the adrenal glands yielded no consistent information despite occasional suggestive data such as the apparent destructive action of pepsin (see Fig. 3). No conclusions could be drawn from an experiment like that of Fig. 6. Study of the microscopical preparations fixed and stained by our routine technique was likewise fruitless. We believe that definite results can be obtained only after adrenal-cortical stimulating effects, which were often definitely produced by our extracts, can be evaluated more accurately in hypophysectomized rats. The staining of the lipins of frozen sections could not be done although this method might yield the desired information. Also it is possible that consistent effects might be demonstrated by the use of large groups of hypophysectomized rats.

The behaviour of the spleen

The spleen was weighed in all the animals and in more than half of the experiments the organ was also fixed and sectioned. We were unable to correlate splenic hypertrophy or morphology with extract dosage despite preceding digestion of much of the protein of extract. The injection of enzyme alone (see the section following this) was usually followed by considerable enlargement of the spleen.

Control experiments

The experiments included control groups of rats either untreated or receiving injections of undigested incubated extract in the same dosage as digested extract. Incubation of the extract, at least in alkaline solution, was not shown to affect the action of extract on the ovaries, uterus, adrenals, and thyroid. The injection of the enzymes was found to have no effect on the organs of hypophysectomized rats other than the spleen. The doses used for each group were the following: crystalline trypsin, 1·3 mg., crystalline chymotrypsin, 1·3 mg., crystalline carboxypeptidase, 2·3 mg., Merck's trypsin, 7·6 mg., papain, 4·6 mg., and crystalline pepsin, 2·3 mg. It was estimated that each dose represented about twice the maximum amount injected in any experiment.

DISCUSSION

It appears that Reiss and Haurowitz [1929] were the first authors to investigate the action of proteolytic enzymes on the gonadotrophic effects of anterior pituitary extract. On the basis of rough assays in normal mice they concluded that crude trypsin or jack-meal extract (digesting peptone but not fibrin) destroyed gonadotrophic hormones. Papain was without effect unless KCN was added as an activator; under the latter conditions,

partial destruction (of luteinizing hormone?) occurred. The work of Evans, Simpson, and Austin [1933] agrees with the view that crude trypsin, activated by crude erepsin, destroys luteinizing hormone but not follicle-stimulating hormone provided that their 'antagonistic factor' be considered identical with the former and their 'synergic factor' be assumed to be follicle-stimulating hormone. The last-named was destroyed by pepsin. These findings agree with ours if the assumptions as to identity are accepted.[1] Bates, Riddle, and Lahr [1934] and Riddle, Bates, Lahr, and Moran [1936] concluded that follicle-stimulating hormone is quickly destroyed by purified trypsin as shown by the failure of digested extracts to cause testicular hypertrophy in ringdoves or ovulation in the rabbit. We are unable to reconcile our observations with those of Guyénot and his co-workers [Guyénot, Ponse, and Dottrens, 1934; Guyénot, Vallette, and Dottrens, 1934; Guyénot, Ponse, and Dottrens, 1935]. These authors performed their assays in immature normal guinea-pigs and concluded that digestion of anterior pituitary extract by pepsin destroys luteinizing (crinogenic) hormone rapidly but inactivates follicle-stimulating (auxogenic) hormone slowly. These findings are the exact reverse of ours in the immature hypophysectomized rat and it does not appear probable that the difference in the species of test animal will account for the discrepancy. In one communication [Guyénot, Ponse, and Dottrens, 1934] it was stated that thyrotrophic hormone resists peptic digestion; however, in the report of 1935, Guyénot *et al.* concluded that this is true only very exceptionally. Fevold [1937] stated that both gonadotrophic hormones are destroyed if digested by either trypsin or pepsin although he published no specific data. It should be mentioned that none of the authors cited above used hypophysectomized animals and that histological studies were the exception rather than the rule.

Recently McShan and Meyer [1938, 1939 *a, b*] and Chen and van Dyke [1939] concluded that crude or crystalline trypsin destroys nearly all the luteinizing action of anterior pituitary extract. Both groups of authors used normal as well as hypophysectomized rats and studied the microscopic appearance of the ovaries. In neither set of observations was the extent of digestion determined; moreover, conditions were not optimal in the experiments of McShan and Meyer in 1938 since the authors conducted the digestion at pH 6·5 for only 3·5 hours. The destructive effects of crystalline trypsin on follicle-stimulating hormone were not recognized by these authors, probably because they did not digest their extracts to a great enough extent. Greep [1939], using the same Merck's trypsin employed in the experiments of Chen and van Dyke and in those now reported, demonstrated that digested extract completely maintained

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[1] See Evans, Simpson, Tolksdorf, and Jonsen [1939].

spermatogenesis in adult hypophysectomized rats and yet, unlike control extract, had no effect on either the interstitial cells or the accessory organs. The latest report is by Abramowitz and Hisaw [1939]. The authors' conclusions were based on the changes in weight of the ovaries or seminal vesicles of normal rats. Lacking histological studies, determinations of extent of digestion, and dose-effect curves for their preparations in the test animals, their data do not appear to justify their belief that neither

Table III.   *Survey of results in simplified form*

| | | Gonadotrophic hormone | | |
Enzyme	Protein digested %	Follicle-stimulating action	Luteinizing action	Thyrotrophic action
Crystalline carboxypeptidase	<5 (5 hours)	Unaffected	Unaffected	Unaffected
	5–12 (30–41 hours)	Unaffected or reduced	Reduced	Unaffected
Crystalline chymo-trypsin	18–35	Reduced	Absent	Absent
	65–80	Absent	Absent	Absent
Crystalline trypsin	12–48	Reduced	Absent	Absent
	61–75	Usually absent	Absent	Absent
Merck's trypsin	10	Unaffected	Unaffected	Unaffected
	35–46	Unaffected or reduced	Reduced	Unaffected or reduced
	61–75	Unaffected or reduced	Absent	Usually reduced
Papain	6–31	Unaffected	Unaffected	Unaffected
	60–65	Reduced	Reduced	Unaffected
Crystalline pepsin	10–34	Unaffected or reduced	Reduced ?	Usually absent
	58–80	Absent	Reduced ?	Usually absent

crystalline trypsin nor crystalline chymotrypsin destroys luteinizing hormone more rapidly than follicle-stimulating hormone. Likewise, although the authors may correctly infer that papain partly destroys follicle-stimulating hormone without affecting luteinizing hormone, their data supply no adequate basis for this conclusion.

Guyénot *et al.* believed that autolysis of anterior pituitary tissue is accompanied by destruction of both luteinizing and thyrotrophic hormones but is without action on follicle-stimulating hormone. McShan and Meyer as well as Abramowitz and Hisaw concluded that ptyalin (saliva) destroys follicle-stimulating hormone but not luteinizing hormone.

The results of our investigation are summarized in Table III, which leaves out of account certain exceptions which are described in the preceding text. However, these exceptions appear not to invalidate tentative conclusions which may be drawn from a consideration of this table. All our

data supporting the view that each of the three hormones (follicle-stimu-
lating, luteinizing or interstitial-cell stimulating, and thyrotrophic hor-
mones) can exist independently and confirm the view originally advanced
by Fevold, Hisaw, and Leonard [1930] that follicle-stimulating and luteiniz-
ing hormones can be separated. The particular sample of Merck's trypsin
used can abolish all or nearly all luteinizing and interstitial-cell stimulating
effects[1] of the extract while preserving a large part both of thyrotrophic
hormone and of follicle-stimulating hormone which provokes adequate
secretion of oestrogen as shown by hypertrophy of the uterus and its
epithelium. Provided that more than half of the protein has been digested,
pepsin leaves intact only the interstitial-cell stimulating (luteinizing)
hormone. It is apparent that neither crystalline carboxypeptidase nor
papain specifically destroys any of the three hormones. Crystalline chymo-
trypsin and crystalline trypsin alike destroy all three hormones when about
60% or more of the protein of the extract has been digested. At lower levels
of protein digestion, and infrequently at high levels, a follicle-stimulating
effect alone persists but appears not to be adequate since the expected
accompanying effects on the uterus may be surprisingly slight. The effects
of all the digests on the ovaries are paralleled by similar effects on homo-
logous parts of the testis. We believe the view that luteinizing hormone
may be identical with thyrotrophic hormone, as suggested by some
authors [Jensen and Tolksdorf, 1939], must be rejected on the basis of
our observations. The possibility that the sensitivity of our test for thyro-
trophic hormone in peptic digests is comparatively lower and that, there-
fore, the hormone would not be recognized after partial destruction seems
ruled out by tests of threshold doses of control extract. In such an experi-
ment thyrotrophic hormone could be detected when interstitial-cell
stimulation in the ovaries was present in some but not all the animals.
Moreover, extracts digested by Merck's trypsin could cause a maximal
thyroid stimulation without evidence of any stimulation of interstitial
cells.

It is our opinion that the validity of our results, so far as the destructive
effects of enzymes are concerned, is increased because the doses used are
comparatively large. Control experiments indicate that the minimally
effective dose for acid or alkaline incubated extracts is no more than 0·1
g.-equivalent of fresh gland (0·09 mg. N) in either sex. Thus, in terms of
control extract, the doses used represent 5, 10, 20, 40, or more minimal
doses.

The importance of the extent of digestion in determining the degree
and nature of the response is illustrated in Fig. 7, the curves of which

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[1] These two effects disappear simultaneously as digestion proceeds and appear to be
caused by the same hormone.

represent frank approximations. In the cases of peptic and Merck-trypsin digests, both qualitative effects (follicle-stimulation and luteinization) are present and the ovarian hypertrophy is definite or pronounced if not more than 35–45% of the protein has been digested. If digestion is carried farther, only one qualitative effect remains; simultaneously an effect on ovarian weight is reduced or absent.

It is obvious that the effects of Merck's trypsin are chiefly caused by
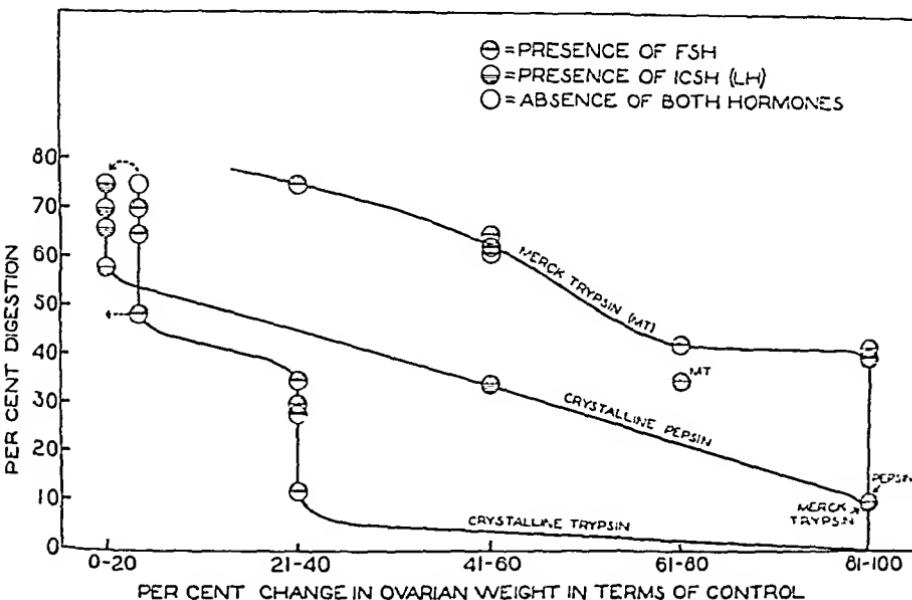
Fig. 7. *Diagram illustrating qualitative ovarian changes and changes in ovarian weight in experiments with three enzymes when extract injected into each rat was equivalent to 1 or 2 g. of fresh hog pituitary. 'Control' refers to groups receiving same doses of extract incubated in solutions at the same pH used for digestion.*

an enzyme or enzymes different from crystalline chymotrypsin or crystalline trypsin (ox). The particular sample of Merck's trypsin which we used appeared to contain only traces of chymotrypsin as estimated by rennet activity. We are aware that the true rennet activity might have been inhibited by other substances in this crude trypsin, but believe that significant amounts of chymotrypsin would, nevertheless, not have yielded only trivial indications of rennet activity. On the other hand, trypsin was found to be responsible for about 30% of the total digestive action as shown by the persistence of about 30% of the original activity after a solution of Merck's trypsin had been boiled in 0·01 N HCl (0·5 mg. per c.c.) and then chilled. Experiments in which, after the addition of a weighed amount of crystalline trypsin to a solution of Merck's trypsin, the activity of the mixture in digesting casein was determined before and after boiling,

showed that the added trypsin could be accounted for quantitatively. Therefore no trypsin-inhibitor was present and the estimate of trypsin present was accurate. All the above data confirm the belief that the proteolytic activity of Merck's trypsin depends to a major extent on the presence of enzyme(s) other than trypsin or chymotrypsin[1] (ox).

It is well known that enzymatic reactions are highly specific and depend on the chemical constitution of the substrates. A great advance in our knowledge of the nature of the linkages attacked by proteolytic enzymes is based on the discovery of the carbobenzoxy method of Bergmann and Zervas [1932]. This method of synthesis of polypeptides enabled the authors to prepare synthetic substrates of their choice. Thus they showed that crystalline trypsin, which differs from chymotrypsin in its inability to clot milk, is active in the presence of an arginine residue as in benzoyl-arginine-amide, whereas chymotrypsin acts upon a tyrosine residue as in benzoyl-tyrosyl-glycine-amide. Neither of these two enzymes will affect the substrate of the other. Furthermore, the substitution of the arginine or tyrosyl groups by other amino acids renders the substrates resistant to both enzymes. Pepsin attacks carbobenzoxy-*l*-glutamyl-*l*-tyrosine [Bergmann and Fruton, 1937; Bergmann, Fruton, and Pollock, 1937; Fruton and Bergmann, 1938].

The concept that enzymatic activity depends on specific chemical groups and configurations of the substrates applies to natural proteins as well as to the synthetic peptides, as shown by the digestion of casein by trypsin and chymotrypsin. Northrop and Kunitz [see Northrop, 1939] found that the addition of trypsin to a casein solution previously digested by chymotrypsin to a maximum, or of chymotrypsin to casein previously digested by trypsin, resulted in a marked increase in hydrolysis. It is therefore certain that the two enzymes split casein at different linkages.

In the light of what has just been stated concerning the specific substrates required for some of the enzymes used by us it is possible that luteinizing and thyrotrophic hormones require the presence of the following intact linkages: arginine amide and tyrosyl (or phenylalanyl) glycine amide. Thyrotrophic hormone, in addition, possibly is inactivated if the glutamyl-tyrosine bond has been ruptured. The glutamyl-tyrosine linkage also may be essential for the action of follicle-stimulating hormone. However, this hormone also is largely destroyed if a large part of the protein is hydrolysed either by chymotrypsin or by trypsin. It is possible that the resistance of follicle-stimulating hormone in the earlier stages of

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[1] The amylase activity of Merck's trypsin is very slight, as indicated by the finding that 0·13 mg. was the minimum amount which hydrolysed (disappearance of iodine reaction) 1·0 c.c. of a 0·5% starch solution in 20 minutes at 36° C.

digestion by chymotrypsin or trypsin depends upon the survival of part of the arginine amide and tyrosyl glycine amide linkages which, as digestion proceeds farther, are split so that all or nearly all the follicle-stimulating activity disappears. It is necessary to emphasize that a great number of other amino-acid linkages, possibly just as specifically attacked by pure enzymes, have not been investigated. Obviously, also, the linkages mentioned on the basis of available knowledge may not be essential so far as hormone action is concerned. Although there is evidence that carbohydrate may be an important or an essential component of follicle-stimulating hormone [McShan and Meyer, 1938, 1939 *a*, *b*; Abramowitz and Hisaw, 1939; Evans, Fraenkel-Conrat, Simpson, and Li, 1939], there can be no question that protein or polypeptide must be intact since the purely proteolytic enzyme pepsin readily destroys follicle-stimulating hormone. If this hormone be considered a glycoprotein, these results are not contradictory.

Our success in obtaining specific ovarian effects has permitted us to make some observations that appear to have an important bearing on the site of oestrogen formation by the ovary. Allen has long contended that this function should be ascribed to the granulosal elements. In a recent review of the literature Corner [1938] upholds the view that the thecal cells are the secreting element. Mossman [1937] relates the occurrence of oestrus in the pocket-gopher, *Geomys bursarius*, to the extensive development of the 'thecal gland' which is seen at this time. Parkes [1926] and Schmidt [1936] have studied the ovaries of mice and guinea pigs after X-ray treatment and concluded that the interfollicular tissue resulting from this treatment produces oestrin. Evans, Simpson, and Pencharz [1937] found that the repair of the ovarian interstitial tissue by their ICSH fraction was not accompanied by any uterine growth. As shown in the text, peptic digests of pituitary gonadotrophic extract may cause extensive development of the interstitial tissue. They also produced a marked hypertrophy of the thecal cells (Plate III, Fig. 41). Plate II, Fig. 26, Plate III, Fig. 41, Plate V, Fig. 56, and Plate VI, Fig. 66 are based on an animal representative of this type of treatment and show (*a*) absence of follicular growth, (*b*) extensive hypertrophy of the thecal cells and of the interstitial cells, and (*c*) the concomitant absence of an oestrogenic effect on the uterus. From this we conclude that the interstitial cells of the rat do not secrete oestrogen and that the hypertrophied thecal cells are also not concerned in this process. We have observed instances in which some growth of the follicles may occur after treatment with tryptic digests with little or no growth of the uterus. On the other hand, growth of the uterus has never been observed in our animals in the absence of definite follicular development.

SUMMARY

The effects of digestion by proteolytic enzymes on hormones in extracts of fresh hog pituitary glands were investigated by comparing the actions of digested and incubated control extracts in hypophysectomized immature female and male rats. In the preparation of the extracts by a single method, two objectives were sought: (1) to avoid as far as possible any denaturation of protein, and (2) to employ a purified extract which, however, retained the various hormones of the anterior pituitary. Tests with control extracts indicated that gonadotrophic hormones and thyrotrophic hormone were clearly present. No satisfactory data on the hormone stimulating the adrenal cortex were obtained.

Crystalline carboxypeptidase, crystalline chymotrypsin (ox), crystalline trypsin (ox), a commercial trypsin (E. Merck), purified papain, and crystalline pepsin (hog), were used as enzymes. The percentage of protein digested was often of decisive importance in determining destruction or survival of the three hormones (follicle-stimulating, luteinizing or interstitial-cell stimulating, and thyrotrophic) which, it is believed, can exist independently.

Crystalline carboxypeptidase and papain were found to have no specific effect but probably caused some general destruction. Crystalline chymotrypsin and crystalline trypsin destroyed luteinizing hormone and thyrotrophic hormone; they impaired the action of follicle-stimulating hormone and often destroyed it at high levels of digestion. Merck's trypsin destroyed luteinizing hormone only, whereas crystalline pepsin destroyed folliclestimulating and thyrotrophic hormones. Homologous parts of the testes of immature hypophysectomized males were stimulated by gonadotrophic hormones surviving digestion.

The results are discussed in respect of: (1) previous work, (2) specific activities of enzymes, and (3) the site of oestrogen secretion.

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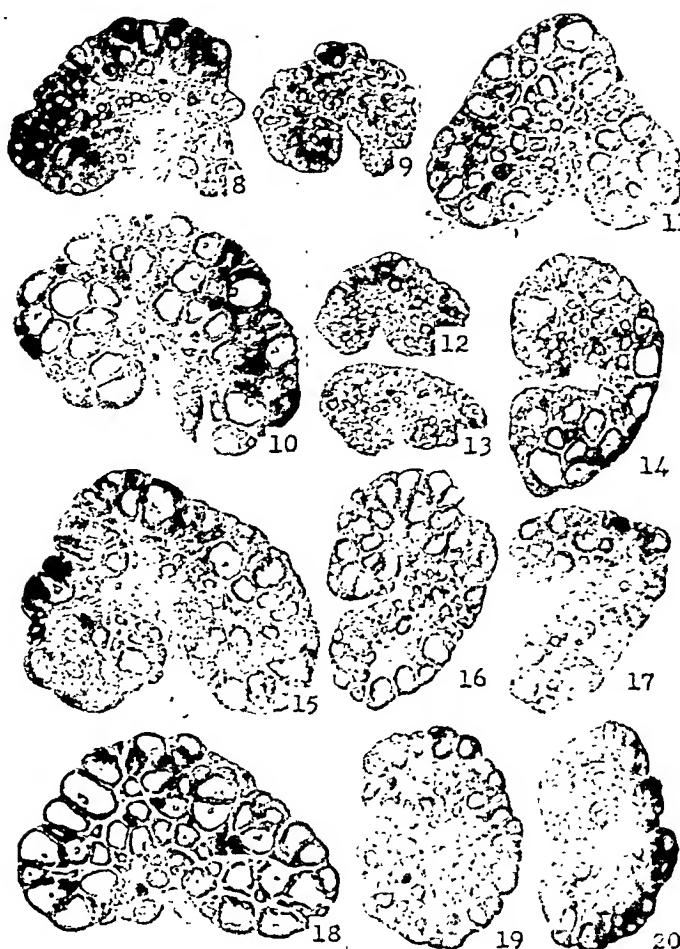
OVARIES (Figs. 8–20). × 10

Fig.	Fresh wt.	Extract			Other figs. of same group
		Dose	Enzyme	Digestion	
	mg.	g.-equir.		% protein	
8	21·8	—	Normal control	—	23
9	10·1	—	Hypo. control	—	22
10	65·4	2·0	Alkaline control	—	5c, 7b
11	33·7	2·0	Cryst. carb. polypep.	10	—
12	9·4	2·0	Cryst. chym. tryp.	29	63, 81
13	8·1	2·0	Cryst. trypsin	35	64, 82
14	22·7	2·0	Merck trypsin	35	40, 42, 61, 65, 83
15	58·8	1·0	Alkaline control	—	6b
16	20·4	1·0	Cryst. chym. tryp	45	47, 61, 87
17	18·2	1·0	Cryst. trypsin	45	45, 62, 88
18	33·2	1·0	Merck trypsin	41	43
19	20·8	8·0	Cryst. chym. tryp.	60	5, 89
20	18·4	8·0	Cryst. trypsin		4, 90

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PLATE II

OVARIES (Figs. 21–32). × 10

		Extract			
					Other figs. of same group
Fig.	*Fresh wt.*	*Dose*	*Enzyme*	*Digestion*	
	mg.	g.-equiv.		% protein	
21	79·2	10·0	Alkaline control	—	50
22	43·3	10·0	Merck trypsin	62	51
23	44·0	2·0	Acid control	—	54, 59, 79
24	7·2	—	Hypo. control	—	44, 60, 80
25	44·7	2·0	Papain	65	—
26	8·4	2·0	Cryst. pepsin	75	41, 56, 66, 84
27	17·1*	2·0	"	69	55
28	25·2	2·0	"	34	—
29	33·3	0·5	Acid control	—	85
30	11·1	0·5	Cryst. pepsin	58	57
31	73·8	10·0	Acid control	—	—
32	39·8	10·0	Cryst. pepsin	58	86
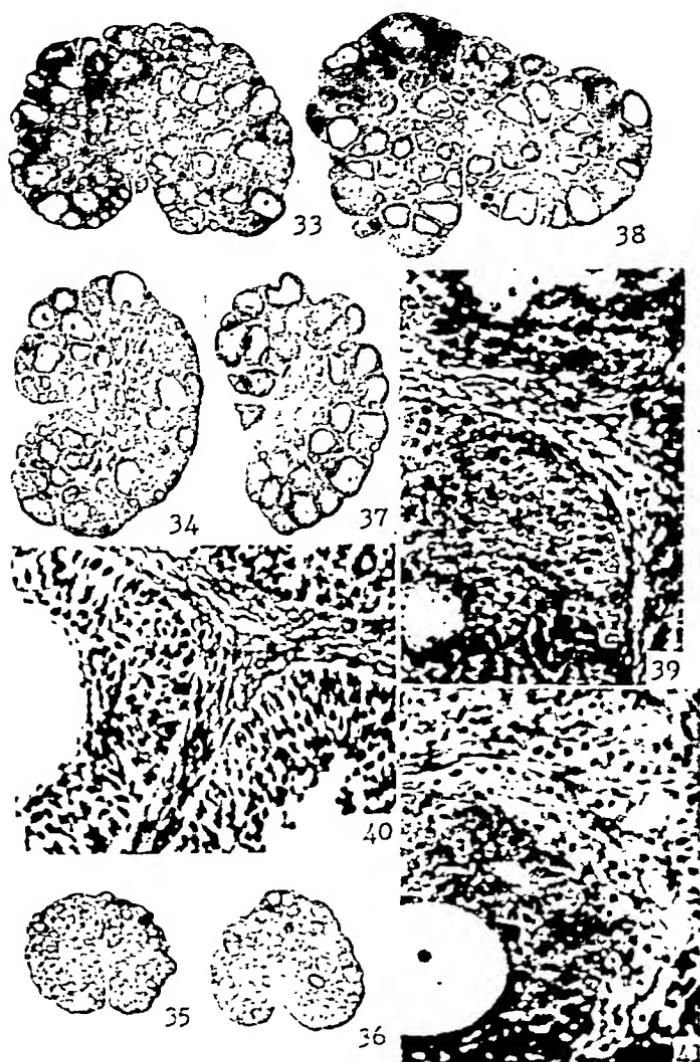
OVARIES (Figs. 33–8). ×10. FOLLICLE WALLS (Figs. 39–41). ×258

Fig.	Fresh wt.	Extract			Other figs. of same group
		Dose	Enzyme	Digestion	
	m.J.	g.-equiv.		% protein	
33	59·0	2·0	Alkaline control	—	—
34	36·7	2·0	Acid control	—	—
35	8·6	—	Hypo. control	—	—
36	10·0	2·0	Cryst. pepsin	63	—
37	32·0	2·0	Merck trypsin	63	—
38	79·2	{1·0 / 1·0	Cryst. pepsin / Merck trypsin	62 / 62	—
39	8·0	—	Hypo. control	—	—
40	25·7	2·0	Merck trypsin	75	34, 42, 63, 67, 83
41	8·4	2·0	Cryst. pepsin	75	2, 36, 44, 84

LUTEIN TISSUE (Fig. 42) AND OVARIAN INTERSTITIAL CELLS
(Figs. 43–9). ×528

Fig.	Fresh wt.	Extract			Other figs. of same group
		Dose	Enzyme	Digestion	
	mg.	g.-equiv.		% protein	
42	28·7	2·0	Merck trypsin	75	14, 40, 43, 65, 83
43	28·7	2·0	"	75	14, 40, 42, 65, 83
44	7·2	—	Hypo. control	—	24, 60, 80
45	20·8	8·0	Cryst. chymotryp.	69	19, 89
46	18·4	8·0	Cryst. trypsin	61	20, 90
47	26·4	1·0	Cryst. chymotryp.	35	16, 61, 87
48	18·2	1·0	Cryst. trypsin	28	17, 62, 88
49	53·2	1·0	Merck trypsin	41	18
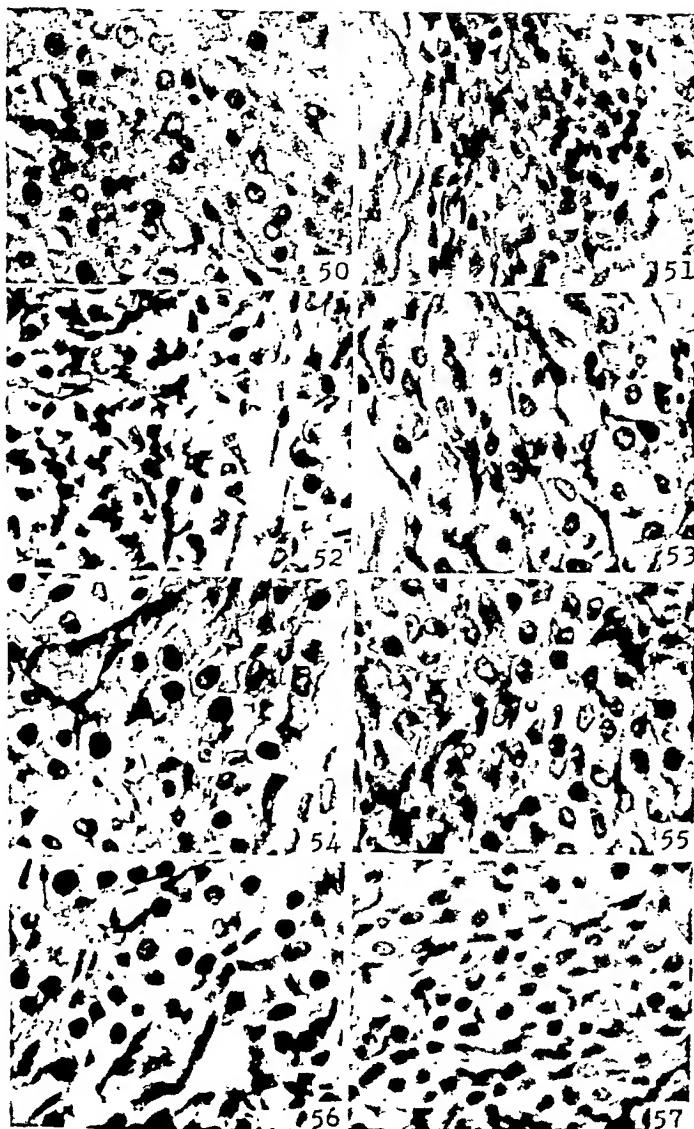
INTERSTITIAL CELLS OF OVARY (Figs. 50-7). × 528

		Extract			
Fig.	Fresh wt.	Dose	Enzyme	Digestion	Other figs. of same group
	mg.	g.-equiv.		% protein	
50	79·2	10·0	Alkaline contrl	—	51
51	43·3	10·0	Merck trypsin	62	52
52	10·1	—	Hypo. contrl	—	?
53	21·5	—	Normal contrl	—	?
54	11·0	2·0	Acid contrl	—	55, 56, 57
55	17·1*	2·0	Cryst. pepsin	62	?
56	8·4	2·0	"	75	54, 41, 66, ??
57	11·1	0·5	"	??	??

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PLATE VI

UTERI (Figs. 58–66). × 660

| Fig. | Fresh wt. | Extract | | | Other figs. of same group |
| | | Dose | Enzyme | Digestion | |
	mg.	g.-equiv.		% protein	
58	60·8	2·0	Alkaline control	—	10, 78
59	63·8	2·0	Acid control	—	23, 54, 79
60	11·1	—	Hypo. control	—	24, 44, 80
61	15·1	1·0	Cryst. chymotryp.	35	16, 47, 87
62	12·6	1·0	Cryst. trypsin	28	17, 48, 88
63	11·6	2·0	Cryst. chymotryp.	80	12, 81
64	12·7	2·0	Cryst. trypsin	75	13, 82
65	111·4	2·0	Merck trypsin	75	14, 40, 42, 43, 83
66	12·5	2·0	Cryst. pepsin	75	26, 41, 56, 84

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PLATE VII
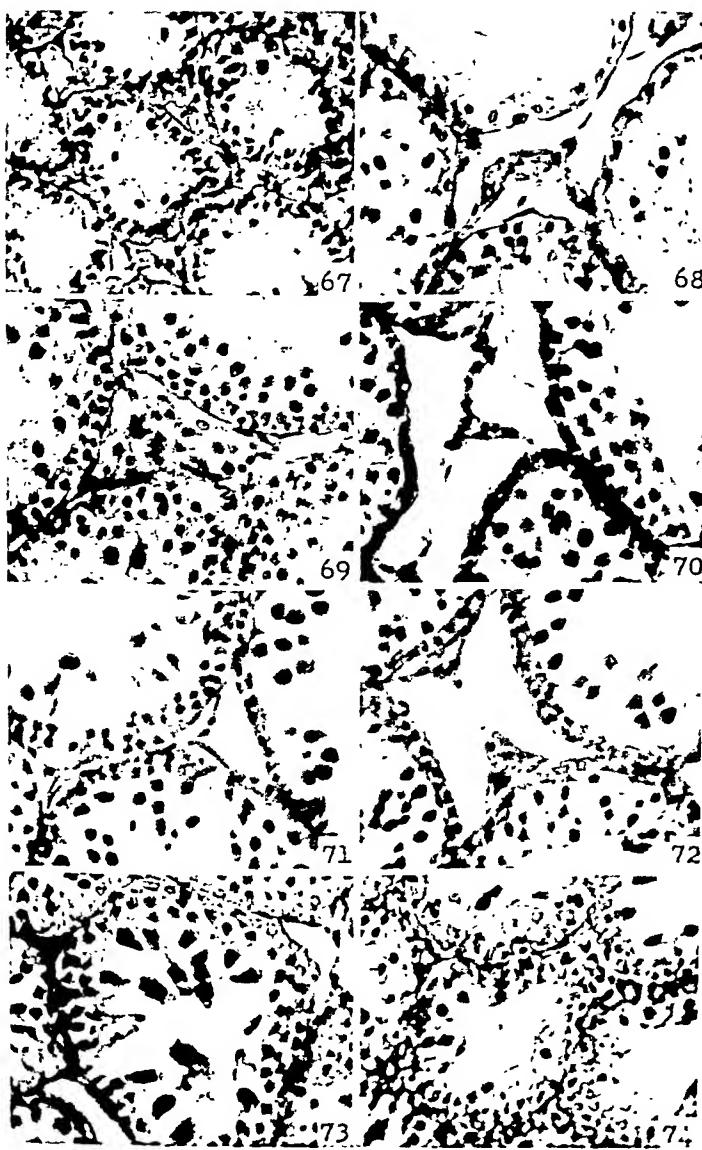
TESTES (Figs. 67–74). × 258

Fig.	Fresh wt.	Extract			Other figs. of same group
		Dose	Enzyme	Digestion	
	m.g.	g.-equir.		% protein	
67	78·2	—	Hypo. control	—	
68	350·6	4·0	Alkaline control	—	
69	104·4	4·0	Acid control	—	
70	336·0	4·0	Cryst. pepsin	6·6	
71	357·4	4·0	Merck trypsin	6·1	
72	341·6	{2·0 / 2·0	Cryst. pepsin	6·1 / 6·6	
73	218·4	4·0	Cryst. chymotryp.	6·5	
74	146·0	4·0	Cryst. trypsin	6·5	

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PLATE VIII
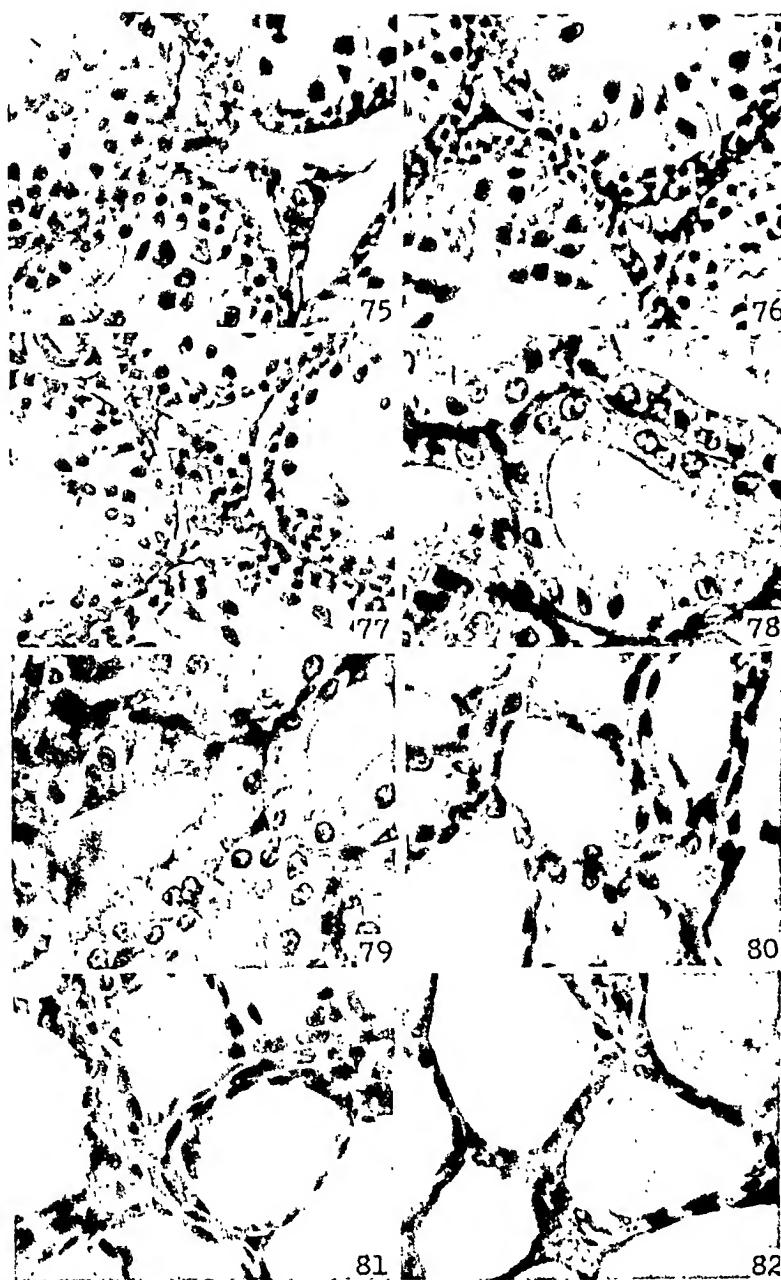
TESTES (Figs. 75-7). ×258. THYROIDS (Figs. 78-82). ×528

Fig.	Fresh wt.	Extract			Other figs. of same group
		Dose	Enzyme	Digestion	
	mg.	g.-equiv.		% protein	
75	579·2	10·0	Alkaline control	—	—
76	449·8	10·0	Merck trypsin	62	—
77	247·6	10·0	Cryst. pepsin	80	—
78	7·0	2·0	Alkaline control	—	10, 58
79	7·5	2·0	Acid control	—	23, 54, 59
80	4·7	—	Hypo. control	—	24, 44, 60
81	4·6	2·0	Cryst. chymotryp.	80	12, 63
82	4·3	2·0	Cryst. trypsin	75	13, 64
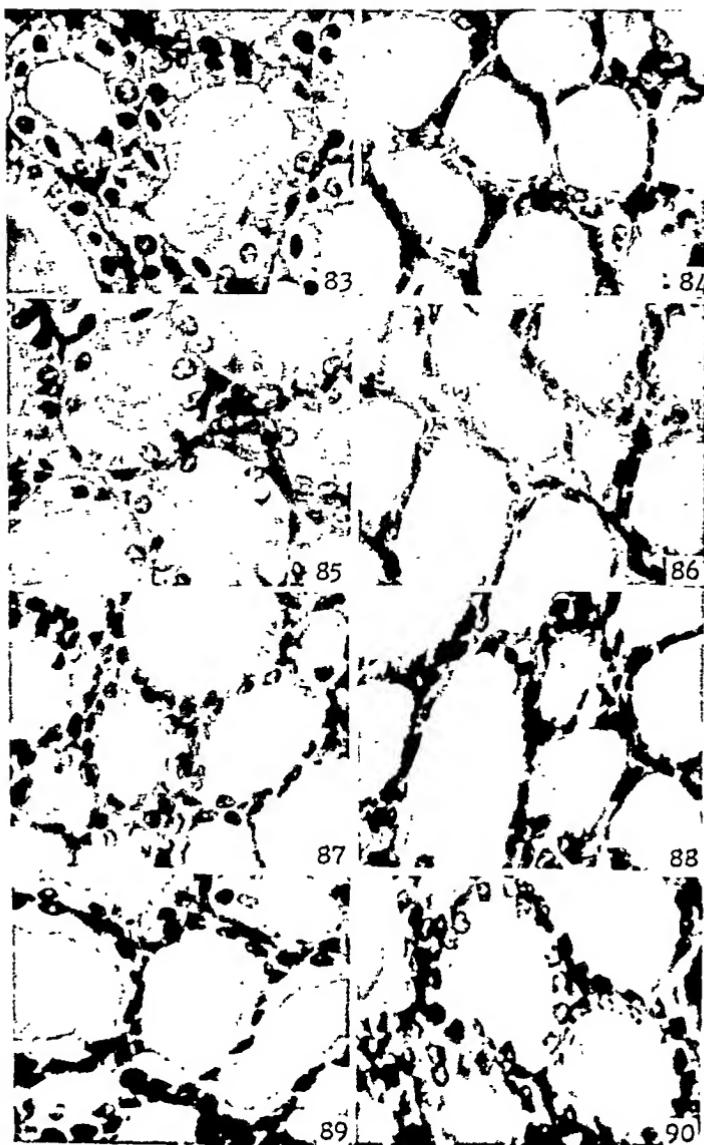
THYROIDS (Figs. 83-90). × 528

| Fig. | Fresh wt. | Extract | | Injection |
		Dose	Enzyme	
	mg.	g.-equiv.		
83	14	20	Merck trypsin	73
84	12	20	Cryst. trypsin	33
85	51	0·5	Acid c nt l	—
86	31	100	Cryst. trypsin	25
87	13	10	Cryst. chymotryp.	5
88	51	10	Cryst. trypsin	25
89	53	80	Cryst. chymotryp.	0
90	50	50	Cryst. trypsin	0

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OBSERVATIONS ON THE METABOLISM OF DOGS MADE PERMANENTLY DIABETIC BY TREATMENT WITH ANTERIOR PITUITARY EXTRACT

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THE observation that a permanently diabetic condition may be induced in dogs by a short period of treatment with anterior pituitary extract [Young, 1937] has been generally confirmed [Campbell and Best, 1938; Houssay and Biasotti, 1938; Dohan and Lukens, 1939; Loubatières, 1939 a, b]. Subsequently it was found that changes in the islets of Langerhans of the pancreas were demonstrable in those animals in which a permanently diabetic condition had thus been established [Richardson and Young, 1938; Campbell and Best, 1938; Dohan and Lukens, 1939]. In the present paper, which is concerned with investigations on the nature of the metabolism of these diabetic dogs, the term 'pituitary-diabetes' will be used to describe the permanently diabetic condition established by such a short period of pituitary treatment, but it must be stressed that there is no intention to imply by the use of this expression that the diabetic condition is one associated with a continued over-activity of the anterior lobe of the pituitary gland. On the contrary, evidence will be adduced that this condition is not associated with any obvious change in the pituitary function of the diabetic animal. It is necessary, however, to use a term which will clearly distinguish the diabetic condition induced in dogs by pancreatectomy, on the one hand, and by a short treatment with anterior pituitary extract, on the other. The terms 'pancreatic-diabetes' and 'pituitary-diabetes' are clearly the most suitable for this purpose, if the particular meanings here attached to them are clear and explicit.

During the past two years observations have been made in this laboratory concerning the metabolism of dogs with pituitary-diabetes, and a number of preliminary observations have been recorded [Young, 1937, 1938 a, b; Marks and Young, 1938; Young, 1939 a, b]. The present communication completes the publication of the data so far obtained in this laboratory, and includes a general discussion on the metabolism of these dogs.

METHODS

Biological

Induction of pituitary-diabetes in dogs. Permanent diabetes was induced in young male dogs, weighing about 10 kg., by the daily administration, for a short period, of crude ox anterior pituitary extract according to the method already described [Young, 1937, 1938 a]. The amount of extract injected daily was progressively increased every few days until the dose administered daily was equivalent to 25 g. of fresh ox anterior lobe tissue. Daily treatment with this amount of extract was continued until a point was reached at which cessation of injections resulted in no diminution of glycosuria. The conditions governing the conversion of the temporary into the permanent form of diabetes, induced by treatment with pituitary extract, have recently been discussed [Young, 1939 a].

In our experience, once the permanent state of diabetes has been produced, the condition shows no sign of remission whatever, but, on the other hand, sometimes tends to increase somewhat in severity with the passage of time. In particular, the ketonuria, which usually falls to a very low level soon after the cessation of pituitary injections, rises again later, and sometimes slowly but progressively increases over a long period of time.

Pancreatectomy was performed in a one-stage operation, the completeness of removal being verified in all cases at post-mortem examination. For 2–3 weeks after operation the dogs were given two daily injections of insulin of sufficient magnitude to depress the glycosuria to a low level when a diet of raw meat and pancreas was fed. When the abdominal wound had completely healed the amount of insulin given daily was gradually reduced to zero over a period of 3–5 days; observations on the untreated depancreatized dogs were usually begun 2–5 days after the administration of insulin had been completely stopped.

Collection of urine. The experimental animals were housed in large metabolism cages, the urine being collected under toluene. As the animals were not catheterized at the end of each 24-hour period, only average values for urine composition over a period of about 7 successive days were considered in most instances.

Experimental diets. During the various experimental periods, the dogs were given weighed amounts of food in one or two portions, at fixed times each day. Any uneaten residue was weighed and the amount of food eaten thus determined. However, in almost every instance the whole of the food offered was rapidly eaten.

The average composition of typical diets is given in Table I. The amount of food ingested daily was, in most instances, so adjusted that the animal

received the equivalent of just over 900 calories. Thus, except in the case of a high carbohydrate diet, the food was approximately sufficient to cover the metabolic needs of the animal, allowing for the loss of sugar in the urine. The horse meat was freed from visible fat and cut into small pieces or minced, to facilitate digestion; in preparing the 'Toronto' diet (Table I) the raw pancreas and raw meat were thoroughly mixed by being minced together with constant stirring. In spite of our attempts to main-

Table I. *Composition and characteristics of typical diets*

Type of diet	Composition	Calories	Protein g.	Fat g.	Carbohydrate g.	
					Preformed	Total available
High protein	1000 g. raw lean horse flesh	932	190	12	10	121
	480 g. boiled lean horse flesh + 90 g. boiled ox liver	901	164	23	4	100
'Toronto'	400 g. raw horse meat + 200 g. raw ox pancreas + 40 g. glucose	739	100	15	46·5	104
	500 g. raw horse meat + 250 g. raw ox pancreas + 50 g. glucose	925	125	19	58	130
High carbohydrate	220 g. wheatmeal biscuit + 90 g. of boiled ox liver	971	55	8	164	196
	300 g. wheatmeal biscuit	1086	36	3	222	243
High fat	50 g. beef suet.	455	0·2	48·5	0	(4·8 ?)*

* Glycerol, which is not considered as forming sugar available for excretion by the diabetic animal (see below).

tain a uniform supply of meat the composition undoubtedly varied quite substantially from time to time, as shown by variations in the nitrogen excretion when apparently the same diet was given (cf. Table III).

Bones were given once or twice a week and vitamin supplements in the form of 'radiostoleum' (A and D) and dried yeast were added to the food once a week. Water was given *ad libitum*, care being taken to anticipate any increase in water intake occasioned by a change of experimental conditions. The animals always had access to rock salt in case the substantial loss of NaCl in the large volume of urine excreted should induce salt depletion.

For the purpose of carrying out sugar tolerance tests or of determining the proportion of ingested sugar excreted in the urine, glucose was given in the solid form—small pellets, which the animals readily ate—or as solution which, in a few instances, was administered by stomach tube. For

sugar tolerance curves the sugar was given when the animal was in a fasting condition, but otherwise it was given in addition to the ordinary food.

Casein, when supplementing a high-fat diet, was kneaded into a dough with a little water, and lightly baked into the form of an appetizing biscuit.

The total daily amount of food was usually divided into two equal portions, one of which was given in the morning at about 10 a.m., and the other at about 5 p.m. In those experiments in which insulin was given, this was administered subcutaneously in two equal doses, one before

Table II. *Excretion of nitrogen in faeces by diabetic dogs*

Dog	Condition	Diet	Period days	Protein fed g./day	Urinary nitrogen excretion g./day	Faecal nitrogen excretion g./day
40	Pituitary dia-betic	High protein	7	164	24·6	1·66
50	,,	High carbo-hydrate	5	42	5·8	0·90
60	Depancreatized	'Toronto Diet'*	7	116·5	17·2	1·44
60	,,	Raw meat+ *liquor pan-creaticus*	2	187	22·15	14·20

* Toronto diet = raw meat+raw pancreas+glucose. See Table I.

each meal. It was of importance to ensure that all the food was eaten immediately after the insulin had been injected in these experiments, in order to obtain constant results regarding insulin requirements on the different diets. Any delay in eating food tended to cause considerable variations in glycosuria, even when constant amounts of food and insulin were given.

The pituitary-diabetic dog, which has its digestive system intact, is able to digest food in a normal manner, and in the early experiments diets containing no digestive enzymes were given to these dogs. The depancreatized dog, however, cannot digest protein and fat to the normal extent owing to the absence of pancreatic enzymes, and in order accurately to compare the metabolic condition of the pituitary-diabetic dog with that of the depancreatized animal, it was necessary to feed both types of diabetic dog on a diet containing an ample supply of pancreatic enzymes. The 'Toronto' diet used in these experiments was that employed by Dr. C. H. Best and his colleagues in the University of Toronto, and described by Campbell and Best [1938]; it contains a large proportion of raw pancreas (Table I). The use of the same type of diet in the two laboratories will facilitate the direct comparison of the results of the two groups of investigators.

The results given in Table II show that the amount of nitrogen excreted

in the faeces of the depancreatized dog receiving the 'Toronto' diet is about the same as that in the faeces of protein-fed pituitary-diabetic dogs. Table III summarizes the figures for average urinary nitrogen excretion by pituitary-diabetic and depancreatized dogs, during periods in which they were receiving ample insulin therapy and could be assumed to be very nearly in nitrogen equilibrium. The body-weight changes in the different groups were certainly very closely similar, and under these conditions we may safely assume that the urinary nitrogen excretion is an indicator of the extent to which nitrogenous material is absorbed from the intestine. The results clearly show that the absorption of protein

Table III. *Urinary excretion of nitrogen by diabetic dogs receiving 'Toronto' diet together with insulin*

Daily food intake	Dogs	Condition	Total no. of days	Average daily nitrogen excretion ·g.	Mean body-weight change kg./day
640 g. of 'Toronto' diet	44 & 50	Pituitary-diabetic	17	14·75	+0·05
,,	60, 65, & 67	Depancreatized	82	14·55	+0·01
,,	44	Depancreatized pituitary-diabetic	32	16·55	0·00
800 g. of 'Toronto' diet	44 & 50	Pituitary-diabetic	66	19·05	+0·05
,,	60, 65, 67, 69, 70, 71	Depancreatized	93	18·70	+0·05
,,	44	Depancreatized pituitary-diabetic	40	19·76	+0·04

from the gut of the depancreatized dog fed on the 'Toronto' diet is almost as great as that for the pituitary-diabetic dog. The results in Table III show that, when 800 g. of 'Toronto' diet were given daily, depancreatized dogs excreted, on the average, 0·35 g./day less nitrogen through the kidneys than did the pituitary-diabetic dogs, although the average body-weight change in the two groups was the same. As the pituitary-diabetic dogs excreted 19·05 g./day of nitrogen, on the average, we can calculate that the nitrogen excretion of the depancreatized dogs was

$$\left(\frac{19\cdot05 - 0\cdot35}{19\cdot05}\right)100 = 98\cdot3\%$$

of that of the pituitary-diabetic dogs. This difference is so small as to be negligible, although in some experiments 3% more food was given to the depancreatized dogs to counteract any such slight difference in power of absorption from the intestine. The fact that ingested glucose is excreted quantitatively, or almost quantitatively, in the urine of the depancreatized dog [cf. Minkowski, 1893; Barker, Chambers, and Dann, 1937; &c.] shows

that in the complete absence of pancreatic enzymes glucose can be almost completely absorbed from the gut of the dog. We have found no evidence that our depancreatized dogs were able to absorb glucose less efficiently than were the pituitary-diabetic dogs.

Respiratory metabolism was determined by means of a closed circuit apparatus designed by one of us (H. P. M.) and described in the Appendix. Average metabolic rates were calculated from the gaseous exchange over periods of 24 hours in some instances, but more frequently over shorter periods. The results were expressed as calories per square metre of body surface per day, using Meeh's formula $S = 0.112 \sqrt[3]{(Body\text{-}weight)^2}$ in calculating the body-surface. During the determinations of metabolic rate no steps were taken to ensure that the dogs were in a 'basal' condition; they were allowed to indulge in their usual activity, as it was hoped thus to obtain conditions approaching those in the metabolism cages in which the dogs normally lived. In those experiments in which glucose was given, the metabolic rate was determined over a period of 2 hours before the administration of glucose, and for one or two periods, each of 2 hours, after the administration.

Chemical

Blood.

Blood sugar was determined by the Hagedorn-Jensen method on 0·1 c.c. of blood obtained from the ear vein.

Urine.

Urinary sugar was determined by titration with Benedict's quantitative reagent; the reagent was frequently checked by titration against polarimetrically standardized solutions of glucose. The sugar content of the urine as determined by Benedict's method was occasionally compared with results obtained by the polarimetric analysis of the amount of sugar in the urine. No discrepancy was ever encountered outside the probable experimental error of the methods, and it was therefore assumed that the only sugar present in the urine in substantial amount was glucose.

Urinary 'ketone bodies' were determined by the Denigès-Van Slyke gravimetric method, the β-hydroxybutyric acid being oxidized with hot dichromate solution [Peters and Van Slyke, 1931]. The results are expressed as 'total acetone'. If the amount present was too small to be accurately determined by the gravimetric method, an approximate determination was made on the basis of the intensity of the Rothera and Gerhardt colour tests.

Urinary nitrogen was determined by Kjeldahl's method.

Daily tests for albumin (sulphosalicylic acid test) were made, in order to detect any kidney damage that might have developed. None was ever

found, although it should be mentioned that a slight but temporary albuminuria was sometimes observed during the preliminary period of anterior lobe injections. The pH of the urine was determined daily in order to detect any development of acidosis.

Foodstuffs.

For analysis, meat was finely minced and biscuit well powdered, the water content being estimated on a sample by drying to constant weight at 100° C. The dried residue was powdered and thoroughly extracted with hot petroleum-ether, the extracted fat being recovered, by evaporation of the solvent, and weighed. The residue left after extraction of the fat was incinerated to constant weight for the determination of ash-content.

Free sugar was extracted by repeated leaching of a suitable sample of minced or powdered foodstuff with 80% alcohol. The extracts were combined and evaporated just to dryness, the solid material thus obtained being dissolved in water and freed from protein by means of the Somogyi [1930] acid-zinc sulphate reagent. The sugar in the protein-free filtrate was determined either by the Hagedorn method or by titration with the Benedict reagent.

Glycogen or starch were determined by a modification of the method of Evans, Tsai, and Young [1931].

Calculation of corrected D/N quotients, &c.

In the present communication the Dextrose/Nitrogen or D/N quotients given are, in every instance, corrected values; that is to say, the average daily intake of preformed carbohydrate has been subtracted from the average daily sugar excretion in the determination of the 'D' for the calculation of 'D/N'. The glycerol portion of fat is not considered as preformed carbohydrate for the purposes of these calculations, as according to Lusk [1928] this does not appear in the urine of depancreatized dogs as glucose. In any case, the fat content of our high-protein diets was so small as to make the correction for the glycerol portion of the fat almost negligible.

After any change of regime, and in particular after the withdrawal of insulin, D/N quotients were not determined for two or three days, in order to allow the animal to accustom itself to the new conditions. This was of especial importance after the withdrawal of insulin, as ab normally high D/N quotients may then be found for two or three days owing to the loss of stored, preformed glycogen [Lusk, 1928].

When 50 g. of glucose were added to the food, the amount excreted in the urine was determined on the basis of the D/N quotients for the days preceding and following the day on which the glucose was given. For

example, in an experiment with dog 50 the urinary sugar on one day during a period of high-protein diet was 102·1 g., nitrogen excretion being 28·5 g. with a corrected D/N of 3·41. The next day 50 g. of glucose were added to the diet, the sugar excretion being 156·1 g., nitrogen being 29·7 g., D/N (corrected for the added 50 g. of glucose) being 3·40; the third day, D = 106·3 g., N = 29·6 g., D/N being 3·43. On the basis of these figures the excretion of the extra sugar was considered to be quantitative. The relevant data for another similar experiment with dog 50 are as follows: first day, D = 84·1 g., N = 25·5 g., D/N = 3·10; on the second day 50 g. of glucose were added to the diet, D being 145·3 g., with N = 31·6 g. and D/N (corrected for the 50 g. of glucose) 2·86. The following day D = 94·1, N = 29·1, D/N = 3·06. On the day on which the glucose was given the D/N would have been 3·08 (the average of the values for the previous and following days), if D had been equal to 152·5 g. The observed value fell short of this by 7·2 g. Therefore the percentage recovery in the urine was $\dfrac{50 - 7·2}{50} \times 100 = 86\%$.

The total available sugar in the diet was calculated on the basis of conversion of protein to sugar in such a proportion that 1 g. of nitrogen is equivalent to 3·65 g. of glucose; the figure for nitrogen used in this calculation was that for the average daily urinary excretion over the period in question; in this way the influence of variations in the composition of the meat was reduced to a minimum. The question of the maximum amount of sugar that can be formed, in the body, from protein has been discussed by Lusk [1928], who concludes from the results of experiments with depancreatized and with phloridzinized dogs that the maximum conversion is expressed by a D/N quotient of 3·65. As the D/N quotients we observed for our animals were not far from this figure, we felt justified in using Lusk's value. It is clear, however, that if the tissues of the depancreatized or the phloridzinized dog are capable of oxidizing any sugar at all, then the urinary D/N quotient may not be an indicator of the maximum amount of sugar that can be formed from protein. Nevertheless, as we ourselves were concerned primarily with the urinary excretion of sugar and nitrogen, it seemed reasonable to use the value of 3·65 in our calculations. In calculating the total available sugar for a diet, no account was taken of the glycerol portion of the (in most instances) small amount of fat in the food.

RESULTS

General

It is convenient to describe the diabetic condition which is exhibited during a period of injections with anterior lobe extract as 'the phase of

temporary diabetes'; if treatment with the extract is of sufficient intensity, the temporary phase of diabetes evolves into the permanently diabetic condition. The conditions under which the temporary may be converted into the permanently diabetic state have recently been discussed [Young, 1939a] and need not be considered here. There is, however, one point that should be mentioned before we consider the general influence of diet on the metabolism of these permanently diabetic dogs: that is the question of ketonuria. In the curves already published, which show the course of events during the period of conversion of the temporary into the permanent diabetes [Young, 1937; 1938a; 1939a], it will be observed that the ketonuria falls to a very low level after the permanently diabetic condition has been established and the injections have ceased. This has led some writers to state that ketonuria is lacking in this type of diabetic condition [cf. Dodds, 1939]. However, figures were published in 1938 showing that when pituitary-diabetic dogs received a raw meat diet, 1·0–13·0 g. of 'total acetone' were excreted each day [Young, 1938a]. In our experience the initial very slight ketonuria of the pituitary-diabetic dog may not increase for some weeks if these dogs are fed on a liberal mixed diet during and after the treatment with pituitary extract. Nevertheless, the ketonuria invariably rises when the animals receive a liberal diet consisting of raw meat only for some weeks, and does not return to the original low level if a restricted mixed diet is again given. In fact a substantial ketonuria is generally maintained unless a high-carbohydrate or a high-fat diet is given (*vide infra*). If such antiketonuric foods are replaced by a normal mixed or high-protein diet, ketonuria again increases. In his classical investigations on the nature of the diabetes which follows extirpation of the pancreas, Minkowski [1893] found that the excretion of acetone, acetoacetic acid, and β-hydroxybutyric acid did not usually follow removal of the pancreas. In a large series of depancreatized dogs he observed the presence of these substances in the urine of five animals only [loc. cit., p. 182]; two of these animals suffered from severe vomiting, and at post-mortem examination were found to have lesions of the gastro-intestinal tract. The urine of these two dogs gave a positive test with ferric chloride towards the end of the first week after the operation. In the other three animals the presence of β-hydroxybutyric acid in the urine was not observed until the pancreatic diabetes was of 2–3 weeks standing, by which time the animals had become severely emaciated. Our own depancreatized dogs were not comparable with those of Minkowski, in that our animals received insulin therapy for some time after removal of the pancreas, at least until the incision had completely healed. When insulin was withdrawn, ketonuria at once developed. In a footnote to his paper [loc. cit., p. 182] Minkowski mentions that traces of acetone are frequently found

in the urine of diabetic animals, when acetoacetic acid and β-hydroxy-butyric acid are not found; in general it seems that only in a very small proportion of instances were these two acids found by him, and even in those in which they were detected, the amounts were not very great, being 4 g. per 24 hours in one case, and 0·5–2 g. in another. It seems, therefore, that Minkowski's depancreatized dogs resembled our pituitary-diabetic dogs, in that ketonuria of any severity was found only in those cases in which the diabetic condition was of relatively long standing. The very slight ketonuria found in our pituitary dogs immediately after the permanently diabetic condition had been established, is paralleled by Minkowski's observations with his depancreatized dogs. The similarity, under some conditions, of the depancreatized dog and the pituitary-diabetic dog, in this respect, might not have been realized if observations on our own depancreatized dogs, which had been treated with insulin for some time after pancreatectomy, had alone been considered.

Another point of general interest that should be mentioned is the fact that the intensity of the diabetic condition induced by treatment with anterior lobe preparations may differ from one animal to another. This has been deduced from the results of metabolic investigations [Young, 1939a] and from histological observations [Richardson and Young, 1938; Richardson, 1939–40]. There is reason to believe that, of the five per-manently diabetic dogs on which extensive investigations have so far been carried out, two were less intensely diabetic than the others [cf. also Campbell and Best, 1938; Dohan and Lukens, 1939]. The results in the present paper support the idea that dogs 40 and 50 were less intensely diabetic than others of our pituitary-diabetic animals.

Experiments with high-protein diets

Table IV summarizes representative data for the excretion of glucose and ketone bodies by protein-fed diabetic dogs not receiving insulin. In most instances results for many experimental periods were obtained, but as the publication of these figures would serve no purpose other than that of confirming the representative results given in Table IV, they are omitted.

D/N quotients, &c. The figures for the corrected D/N quotients for the pituitary-diabetic dogs (Table IV) show that substantial differences exist between different animals in this respect, the extreme values being 3·09 for dog 40, and 3·95 for dog 44. Dohan and Lukens [1939] have also observed high quotients for pituitary-diabetic dogs receiving meat. Values approaching 4·0 and even slightly exceeding this figure were obtained at different times with three of the pituitary-diabetic dogs, but not with dog 40. As indicated by the relatively low D/N quotient and also by the

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somewhat mild ketonuria, this dog appeared to be less severely diabetic than the other animals in this group. The high D/N quotient of 3·95 observed in the case of dog 44 deserves some consideration. If a D/N quotient of 3·65 indicates the maximum amount of sugar which can be formed from the protein [cf. Lusk, 1928], the remaining 0·3 sugar per g. nitrogen must come from other sources. Over the total period of 43 days, this excess of sugar eliminated amounts to 294 g. It cannot arise from the preformed carbohydrate in the diet, since this has already been allowed for, and the total amount of fat in the diet would provide only 110 g. of glycerol for conversion into sugar. The remaining 184 g. must therefore

Table IV. *Average data relating to diabetic dogs untreated with insulin*

Condition	No.	Diet	Period weeks	Urine vol. c.c./day	Glucose excretion g./day	D/N (Corrected)	Ketone excretion g./day	Initial body-weight kg.	Body-weight change kg./day
Pituitary-diabetic	28	Meat	5	1550	91·8	3·40	0·70	10·2	0·00
	40	Meat	6	1450	89·2	3·09	0·28	8·3	0·00
	44	Meat	6	2950	95·5	3·95	5·01	8·8	0·00
	44	'Toronto'	2	3965	132·6	3·71	6·73	8·1	+0·02
	50	Meat	12	1600	91·0	3·45	1·80	8·2	0·00
	50	'Toronto'	2	1950	100·9	3·16	1·56	7·2	−0·02
	51	Meat	7	2750	102·9	3·69	4·03	7·2	0·00
Depan-creatized	60	Raw meat + liquor pancreaticus	—	1550	86·4	3·50	0·45	10·4	−0·12
	60	Raw meat	—	2000	91·2	3·47	2·96	8·9	−0·07
	60	'Toronto'	—	2380	87·6	2·91	4·69	8·4	−0·13
	69	'Toronto'	—	2000	102·3	3·40	2·65	7·5	−0·28
	71	'Toronto'	—	2400	125·1	3·37	4·31	9·2	−0·16

come either from the tissues, from fatty acid, or from protein, and in the latter case the assumed maximal quotient of 3·65 must be incorrect. If the sugar arose from the mobilization of tissue glycogen, it would necessitate the presence initially of this substance to the extent of at least 2·0% of the total body-weight.

Rather unexpectedly the depancreatized dogs also exhibited D/N quotients of the same order as those for the pituitary-diabetic dogs, these values being definitely higher than the classical figure of 2·8, deduced by Minkowski [1893] from the results of his experiments in which depancreatized dogs were fed on meat. However, as Macleod [1928] and others have observed, the D/N quotient varies with the general condition of the animal, higher values being obtained with well-nourished dogs. Moreover, as Soskin [1940] has recently pointed out, the figures for the sugar and nitrogen excretion of depancreatized dogs, which Minkowski employed to establish the D/N quotient of 2·8 for meat-fed depancreatized dogs, were chosen quite arbitrarily; the actual values varied considerably with the general condition of the animal. Our depancreatized animals were in good

condition at the time when insulin was withdrawn and had suffered no great emaciation at the time the tests were made, and it is not therefore surprising that D/N quotients higher than the value of Minkowski were regularly observed. On the whole the D/N quotients for the depancreatized dogs fell slightly below those for the pituitary-diabetic animals, although in view of the variation from animal to animal it would be unwise to assume that any highly significant difference existed in this respect. It is of interest to recall that Langfeldt [1920] found D/N quotients greater than the Minkowski value, with meat-fed dogs which had been made diabetic by partial extirpation of the pancreas. These animals were only slightly or not at all diabetic immediately after the operation, and were able to survive for some time in good condition; later, however, when the diabetic condition developed or became exacerbated, the D/N quotient on a meat diet rose to values well above 3·0. It is therefore possible that such high D/N quotients are met only when the diabetic animal has remained for some time in a reasonably good state of health, in spite of the diabetic condition, and its tissues have thus become accustomed to the abnormal metabolic conditions [cf. Petrén, 1924]. Our pituitary-diabetic dogs possess, in common with Langfeldt's partially depancreatized dogs, ability to survive in good condition in spite of being diabetic for a long time; our depancreatized dogs differ from those of Minkowski and resemble more the dogs used by Langfeldt in being maintained by insulin therapy in a mildly diabetic condition for some time after operation, the more intensely diabetic condition being allowed to develop only after some weeks.

The ketonuria of the pituitary-diabetic dogs varied from time to time and from animal to animal, and, in general, did not obviously differ from that of depancreatized dogs on a similar high-protein diet.

The most striking difference between the two groups of dogs was seen in the changes in body-weight on the meat diet. While the pituitary-diabetic dogs were able to maintain body-weight on a high-protein diet and to live in good condition for many months without insulin therapy, provided that sufficient food was given, the depancreatized dogs invariably lost body-weight rapidly and died within 1–3 weeks, unless insulin therapy was renewed. The figures for body-weight change given in Table IV illustrate this difference between the two groups.

Sugar tolerance and insulin sensitivity. When 50 g. of glucose were ingested by a pituitary-diabetic dog which had fasted 18 hours, sugar-tolerance curves of the type illustrated in Fig. 1a were obtained. These were similar to those observed with depancreatized dogs under similar conditions.

When 5 units of insulin were administered subcutaneously to pituitary-

diabetic dogs which had remained unfed for 18 hours, a substantial response was obtained (Fig. 2), much greater than that found with a dog
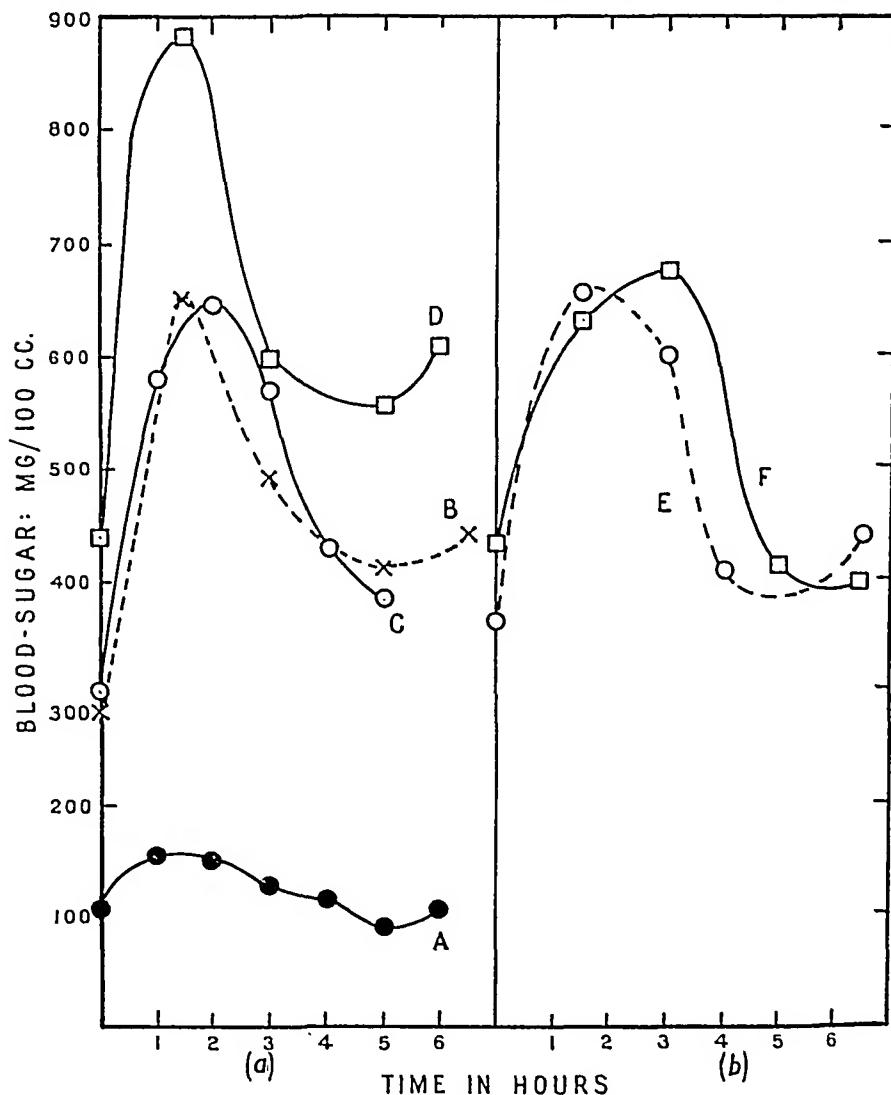
FIG. 1. Blood-sugar response when 50 g. of glucose were administered by mouth to fasting dogs.

(a) Curve A. Average response for 5 normal dogs.
    Curve B. Pituitary-diabetic dog 44.
    Curve C. Depancreatized dog 60.
    Curve D. Pituitary-diabetic dog 51.
The above curves were obtained at a time when the animals had been receiving a high-protein diet.
(b) Curve E. Average response for three pituitary-diabetic dogs which had previously been receiving a high-protein diet.
    Curve F. Response for pituitary-diabetic dog 50 at a time when the diet contained a high proportion of preformed carbohydrate.

which was receiving pituitary injections during a period of temporary diabetes [cf. Benedetto, 1933; Young, 1939a]. There is therefore no reason to believe that these animals are at all resistant to the immediate hypo-

glycaemic effect of administered insulin, a conclusion in agreement with the observations of Dohan and Lukens [1939].

Recovery of glucose added to the diet. When 50 g. of glucose were added
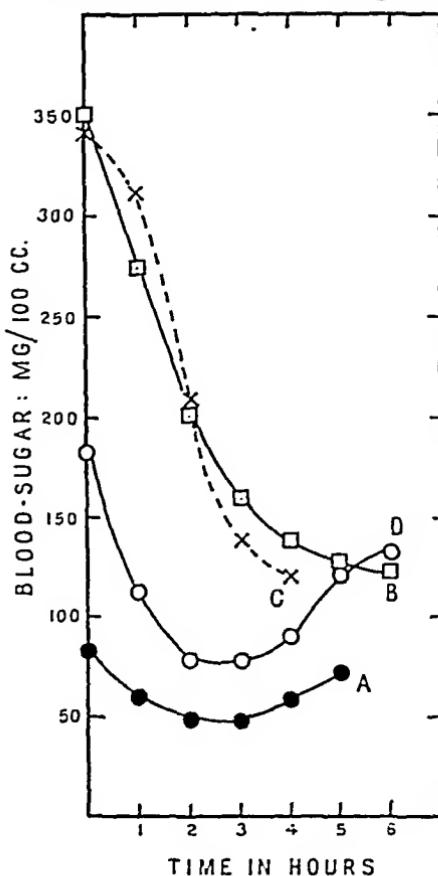
FIG. 2. Blood-sugar response when 5 units of insulin were administered subcutaneously to fasting dogs.

Curve A. Average response for 3 normal dogs.
Curve B. Average response for three pituitary-diabetic dogs previously receiving a high-protein diet.
Curve C. Response for dog 50 when the diet had contained a high proportion of carbohydrate.
Curve D. Response for dog 44 after receiving a high-fat diet for 1½ weeks.

to the food of pituitary-diabetic dogs receiving a meat diet, most of the extra sugar was excreted, as indicated by the constancy of the corrected D/N quotients [cf. Young, 1938a]. The results, which are summarized in Table V, varied from time to time, but on the average 90% of the ingested 50 g. of glucose was recovered as extra sugar in the urine.

Respiratory quotient and metabolic rate. The respiratory quotient (R.Q.) of short-fasting pituitary-diabetic dogs on a meat regime was significantly below that of normal dogs under similar conditions (Table VI), and slightly higher than that of depancreatized animals, though in this case the dif-

Table V. *Excretion of extra glucose by pituitary-diabetic dogs when 50 g. of glucose are added to a high-protein diet*

Dog	Diet	Number of observations	Minimum amount excreted %	Mean amount excreted %
44	Raw meat	4	73	86
44	Raw meat	2	72	86
50	Raw meat	9	72	89
50	'Toronto'	1	—	100
51	Raw meat	1	85	96

Weighted mean = 90%

ference was not significant. When 50 g. of glucose were ingested after a short fast, the R.Q. fell rather than rose, although the fall was not of significance on the basis of our limited number of observations; there was, in any case, no rise.

The figure obtained for the metabolic rate of normal dogs under the conditions of our experiments (Table VI) was about twice the generally

Table VI. *Respiratory data for diabetic dogs*

	Normal dogs		Pituitary-diabetic dogs		Depancreatized dogs	
	No. of observations	Mean value	No. of observations	Mean value	No. of estimations	Mean value
R.Q.	30	$0\cdot755\pm0\cdot010$*	25	$0\cdot700\pm0\cdot012$	6	$0\cdot678\pm0\cdot017$
Rise of R.Q. after 50 g. glucose	20	$0\cdot120\pm0\cdot020$	9	$-0\cdot033\pm0\cdot017$	6	$0\cdot000\pm0\cdot040$
Metabolic rate (Cal./sq. metre/ day)	9	1406 ± 60	9	1503 (a)	6	1700 ± 45
			6	1712 (b)		
			4	1574 (c)		
			19	1580 ± 75 (d)		

(The dogs were previously receiving a meat diet without insulin, but fasted 18 hours before each experiment)

* Standard deviation of the mean.
(a) Observations on dog 44.
(b) ,, ,, 50.
(c) ,, ,, 51.
(d) Log. mean of observations on pituitary-diabetic dogs.

accepted basal metabolic rate for dogs [cf. Kunde and Steinhaus, 1926]. The metabolic rate of the pituitary-diabetic dogs, determined under similar conditions, was about 12% greater than that of the normal animals, though the difference is barely significant on the basis of the recorded number of observations. As was to be expected [Hédon, 1927] the metabolic rate of the depancreatized dogs was, on the other hand, substantially above normal, being over 20% greater than that of the control dogs, the

difference having a high statistical significance in this instance. Hédon and Loubatières [1939] have recently found that the basal metabolic rate of a pituitary-diabetic dog was, unlike that of depancreatized dogs, not increased above normal, and although we did find on the average a slight rise for the metabolic rate of our pituitary-diabetic dogs, the rise was nevertheless smaller than that observed for the depancreatized animals.

Insulin requirement. Table VII summarizes the results from experiments in which pituitary-diabetic and depancreatized dogs were given two daily injections of insulin immediately before food. The food in these experiments consisted of the mixture of raw meat, raw pancreas, and glucose in the proportion of 10 : 5 : 1, designated as the 'Toronto' diet.

In Table VII figures are given for the number of grammes of glucose retained for each unit of insulin administered, in different experiments. The calculation of the amount of glucose retained is based on the average nitrogen excretion during the experimental period and thus allows for the slight variations in the protein intake during the course of the period. It will be seen from the table that the number of grammes of glucose retained per unit of insulin administered did not significantly alter with slight changes in body-weight.

From the data in Table VII it appears that dog 44 required more insulin to control glycosuria than did any of the depancreatized dogs we have examined. When a total of 800 g. of food was given daily to this pituitary-diabetic animal, 60 units of insulin were required to control the glycosuria, whereas the depancreatized dogs required only 20–30 units per day under similar conditions. With the depancreatized dogs, 3·5–6 g. of glucose were retained for each unit of insulin injected, whereas only about 2 g. of glucose were retained for each unit of insulin administered to pituitary-diabetic dog 44.

When the pancreas was subsequently removed from pituitary-diabetic dog 44 the insulin requirement was apparently slightly diminished, falling to about 50 units/day when 800 g. of food were given daily; concurrently, the number of grammes of glucose retained per unit of insulin rose slightly to about 2·7. This apparent change in insulin requirement is so slight as to cast doubt on its reality, but in those experiments in which 800 g. of food were given daily, the results were sufficiently constant to encourage the belief that a small but significant diminution in insulin requirement had resulted from pancreatectomy in this instance. On the other hand, the results of those experiments in which pituitary-diabetic dog 44 received 640 g. of food each day did not provide satisfactory support to the idea that any change in insulin requirement accompanied the removal of the pancreas, although the failure to demonstrate a difference may be due to the fact that the dog was much heavier when the insulin requirement was

determined after pancreatectomy than before the operation (11 kg. compared with 7·6 kg.). One can, however, draw no final conclusion as to whether or not a significant diminution in insulin requirement was asso-

Table VII. *Insulin requirement of diabetic dogs receiving meat+sugar ('Toronto') diet*

Condition of dog	Number and sex	Period	Insulin given	Average sugar excretion	Average nitrogen excretion	Average sugar retention g./unit of insulin	Initial body-weight	Average body-weight change
		days	units/day	g./day	g./day		kg.	kg./day
(a) 640 g. of food/day.								
Pituitary-diabetic	41♂	10	40	9·7	14·7	2·2	7·6	+0·03
	50♂	7	30	4·3	14·8	3·2	8·1	+0·08
Depancreatized	60♀	7	25	0·6	14·7	4·1	11·0	+0·02
	65♂	7	30	trace	16·6	3·5	10·0	+0·01
		24	25	4·7	15·6	3·9	10·0	+0·01
		35	20	33·9	15·6	3·4	9·85	0·00
	67♀	9	20	17·2	15·3	4·2	6·3	+0·03
Pituitary-diabetic after subsequent pancreatectomy	44♂	8	40	trace	16·2	2·6	11·10	0·00
		24	35	26·2	16·7	2·3	11·05	0·00
(b) 800 g. of food/day.								
Pituitary-diabetic	44♂	10	60	11·0	19·4	1·9	10·8	0·00
		18	60	5·2	18·9	2·0	9·4	+0·04
		10	50	23·7	18·7	2·0	9·9	+0·06
	50♂	10	40	5·4	18·5	2·9	8·1	+0·08
		8	25	21·1	18·1	3·9	7·5	+0·06
Depancreatized	60♀	10	20	4·2	19·8	6·3	10·9	+0·02
		8	20	5·6	19·2	6·1	8·6	+0·10
	65♂	10	30	17·7	18·8	3·6	9·2	+0·07
		10	25	25·4	18·9	4·0	8·85	+0·02
	67♀	10	25	9·9	18·7	4·6	6·7	+0·03
	69♂	8	25	10·0	19·3	4·6	7·8	+0·02
	70♂	11	30	8·4	17·8	3·8	8·3	+0·03
		7	25	19·8	18·6	4·2	7·5	+0·10
	71♂	10	30	9·5	18·6	3·9	9·6	+0·02
		9	30	10·6	17·5	3·7	10·2	+0·07
Pituitary-diabetic after subsequent pancreatectomy	44♂	11	50	trace	19·3	2·6	11·05	+0·05
		7	50	15·2	21·6	2·4	12·65	0·00
		14	30	38·7	19·3	2·9	10·5	+0·03
		8	30	45·7	19·6	2·8	9·8	+0·10

ciated with pancreatectomy in this case, but it is certain that the insulin requirement of pituitary-diabetic dog 44 did not fall to that of the normal depancreatized dogs, as the result of removal of the pancreas. Above all, it is clear that the insulin requirement of dog 44 did not increase as the result of pancreatectomy.

Table VIII illustrates the difference between the responses to a small dose of insulin of depancreatized dogs and of pituitary-diabetic dogs. The daily administration of 10 units of insulin to dog 44 resulted in only a slight diminution in D/N quotient, although the ketonuria was reduced to about one-twentieth of its former value. When a similar dose of insulin was given each day to depancreatized dog 70, the D/N quotient was reduced to about one-half its previous value, while a similar result followed the administration of only 5 units to another depancreatized animal—dog 71. In the latter instance the ketonuria was reduced to about one-hundredth of its former value. These results show that the glycosuria and ketonuria

Table VIII. *Influence of small doses of insulin on glycosuria of male dogs receiving 800 g. of 'Toronto' diet each day*

Type of dog	Number	Insulin given units/day	Sugar excretion g./day	D/N Corrected	Ketone excretion g./day	Initial body-weight kg.	Body-weight change kg./day
Pituitary- diabetic	44	0	132·6	3·71	6·72	8·1	+0·02
		10	113·6	3·04	0·34	9·5	+0·01
Depan- creatized	70	5	91·3	2·49	0·08	8·6	−0·01
		10	90·7	1·76	0·03	8·5	+0·01
	71	0	125·1	3·37	4·34	9·2	−0·11
		5	94·6	1·76	0·05	10·4	−0·07

(Average results for periods of 1–2 weeks.)

of the depancreatized dog are more susceptible to control by the daily administration of a small dose of insulin than are the excretion of sugar and ketones by pituitary-diabetic dog 44.

The results of F. N. Allan [1924] indicate that when different doses of insulin are administered daily to depancreatized dogs receiving a constant amount of food each day, a plot of the logarithm of the number of g. of glucose retained each day against the logarithm of the number of units of insulin administered should give a straight line. We have found this to be so for a fairly wide range of insulin doses, not only with depancreatized dogs (cf. curve B, Fig. 3), but also with pituitary-diabetic dogs (curves A and D). Curve C in Fig. 3 is based on data from pituitary-diabetic dog 44, obtained after removal of the pancreas from this animal. The slope and position of the curve for the depancreatized dog (curve B) in Fig. 3 are both similar to those of curves for others of our depancreatized, but otherwise normal, animals, which we have examined in this respect, but are quite different for those plotted for the data for depancreatized dogs given by Allan [1924]. This difference is not surprising in view of the differences in type of food and general conditions of the two different investigations, but it should be pointed out that our depancreatized dogs apparently required substantially more insulin than those of Allan for

the control of glycosuria. Allan's animals received a raw meat-+-sucrose diet, which did not contain raw pancreas.

The fact that the curves relating to pituitary-diabetic dogs all lie to the right of that for the depancreatized animal in Fig. 3 indicates that the pituitary-diabetic animals required more insulin for equivalent controls
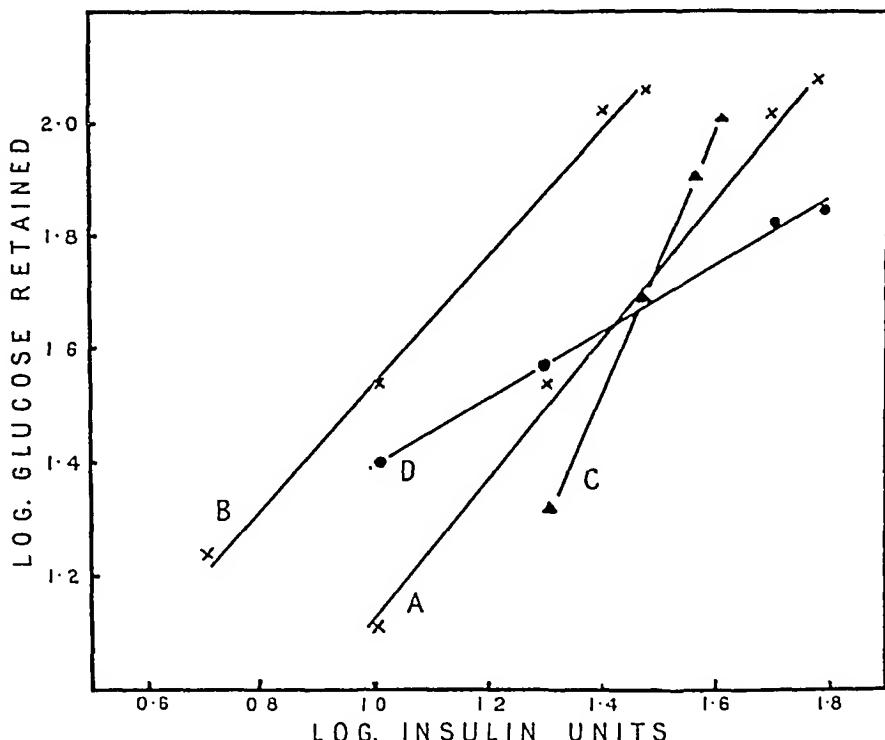
FIG. 3. Curves illustrating the straight line relationship existing between the log. of a dose of insulin administered to diabetic dogs, and the log. of the glucose retained.

Curve A. Pituitary-diabetic dog 44.
Curve B. Depancreatized dog 70.
Curve C. Pituitary-diabetic dog 44 after removal of the pancreas.
Curve D. Pituitary-diabetic dog 40.

of glycosuria, at all levels of insulin therapy. It should be mentioned that curve D relates to a period when dog 40 was receiving a diet of raw meat without pancreas or added sugar, and is therefore not directly comparable with the other curves in Fig. 3, for which the animals received 'Toronto' diet.

The results discussed in this section show that, in some instances at least, the pituitary-diabetic dog requires more insulin for the control of glycosuria than does the depancreatized dog; a similar observation has recently been recorded by Campbell, Keenan, and Best [1939]. Nevertheless, as has already been stressed [Young, 1937; 1938 a, b; 1939 a, b], the pituitary-diabetic dog is able to survive in good condition, and without marked loss of body-weight, if sufficient food is given, in the absence of

insulin therapy; Loubatières [1939 *a*, *b*] has recently made similar observations on a dog made permanently diabetic by treatment with anterior lobe extract. In spite of the fact that the pituitary-diabetic dog is able to survive without insulin-treatment, if insulin therapy is instituted and then suddenly withdrawn the animal may develop a condition resembling diabetic coma and die. If, however, the insulin dosage is slowly diminished, so that complete withdrawal is spread over many days, the pituitary-diabetic dog will survive in good condition, according to our experience,

Table IX. *Influence of high-carbohydrate diet on pituitary-diabetic dogs not receiving insulin*

Dog	Period days	Sugar excretion g./day	Nitrogen excretion g./day	Ketone excretion g./day	Carbohydrate in food g./day	(Carbohydrate)/(Protein) ratio in food	% of preformed carbohydrate excreted	% of total available carbohydrate excreted	Initial body-weight kg.	Body-weight change kg./day
(a) *Biscuit only.*										
40	22	139	8·45	0·15	149	2·8	93	77	6·60	−0·05
44	11	168	8·8	0·41	192	3·5	98	84	7·15	−0·07
50	17	183	5·7	0·29	181	5·0	101	91	7·55	−0·08
50	5	127	5·8	0·57	120	3·3	106	90	7·85	−0·06
						Mean	*99·5*	*85·5*		*−0·06*
(b) *Biscuit + meat.*										
28	14	149	27·5	0·75	60	0·35	250	91	11·10	0
40	10	118	15·7	0·24	97	0·99	122	77	7·05	−0 02
44	9	43	8·8	0·60	30	0·55	143	70	5·65	+0·01

(The figures given are the average daily results for the experimental period in question.)

whereas the depancreatized dog eventually dies unless therapy is again instituted [see Fig. 4 in Young, 1939 *a*].

Experiments with high-carbohydrate diet

Table IX summarizes the results of typical experiments in which pituitary-diabetic dogs received food containing a high proportion of carbohydrate. Two types of high-carbohydrate diet were used; one consisted of dog biscuit alone, the other consisting of dog biscuit together with raw meat.

When dog biscuit alone was given, the whole of the preformed carbohydrate in the food was, on the average, excreted in the urine, but only about 85% of the total available carbohydrate of the diet was lost in this way. The ketonuria was low throughout the experimental period in all instances, but because of the elimination of so much combustible material in the urine, in the form of glucose, the animals rapidly lost body-weight while receiving this food.

When dog biscuit, together with raw meat, was given to these animals, a still smaller proportion of the total potential carbohydrate in the food

appeared in the urine. There can be little doubt that these animals are able to utilize carbohydrate to some degree when a large proportion of preformed carbohydrate is present in the food. No evidence was found that sugar tolerance was altered when dog 50 received a high carbohydrate diet (Fig. 1 *b*), nor was the hypoglycaemic effectiveness of 5 units of insulin altered by these conditions (Fig. 2).

Experiments with high-fat diet

Several pituitary-diabetic dogs tolerated a diet of beef suet alone for some days, and in one instance for several weeks. The ingestion of such

Table X. *Dogs receiving a high-fat diet (beef suet only)*
(No insulin given)

Dog	Period days	Urine volume c.c./day	Glucose excretion g./day	Nitrogen excretion g./day	Suet eaten g./day	Ketone excretion g./day	Initial body-weight kg.	Body-weight change kg./day
40	5	170	3·5	1·0	94	0·24	5·90	−0·02
44	9	1350	4·8	1·8	49	0·54	7·35	−0·06
44	9	500	0·0	0·0	44	0·06	6·60	−0·03
50	3	1150	11·5	3·0	51	2·60	7·05	−0·28
51	3	1700	8·3	3·9	183	3·61	7·35	−0·30
60 (depancreatized)	4	230	9·6	3·2	25*	0·86	7·80	−0·14
	3	330	11·4	4·5	25*†	0·01	7·30	−0·13

* Fat retained as indicated by the difference between that ingested and that excreted in the faeces.
† Together with 50 g. raw pancreas daily.

a diet resulted in a prompt diminution of both glycosuria and ketonuria (Table X), more than 50% of the glycerol of the fat failing to appear as urinary sugar. When 50 g. of glucose were given to dogs receiving this high-fat diet only about 45% was lost in the urine. In two experiments with dog 40 the loss averaged 40%, while in another instance dog 50 lost 48% of the added glucose; in a series of experiments with dog 44 (see below), in which varying amounts of glucose were ingested, the loss varied between 83 and 22% according to the time for which the animal had received the high-fat diet.

Fig. 4 summarizes the data from an experiment in which dog 44 received a high-fat diet for nearly 10 weeks; during this period an average of about 45 g. of beef suet was consumed daily. Immediately before the period of fat-feeding, dog 44 had received a diet of raw meat and cooked liver for some weeks, during which time the glycosuria averaged approximately 100 g./day with a D/N quotient of 3·90 and a mean ketonuria of 4·7 g./day. As shown in Fig. 4, the replacement of the protein food by a high-fat diet was followed by a rapid diminution of glycosuria, which disappeared on the 15th day of fat feeding, and by a fall of ketonuria, which reached a very low level on the 13th day. As shown in this figure the percentage of glucose retained when a test dose was administered

orally steadily increased during the period of fat feeding, until on the 26th day over 75% was retained, this retention being accompanied by a rise in R.Q. Subsequently, on the addition of casein to the fat diet, the sugar tolerance somewhat declined (Fig. 5). Insulin sensitivity, tested by
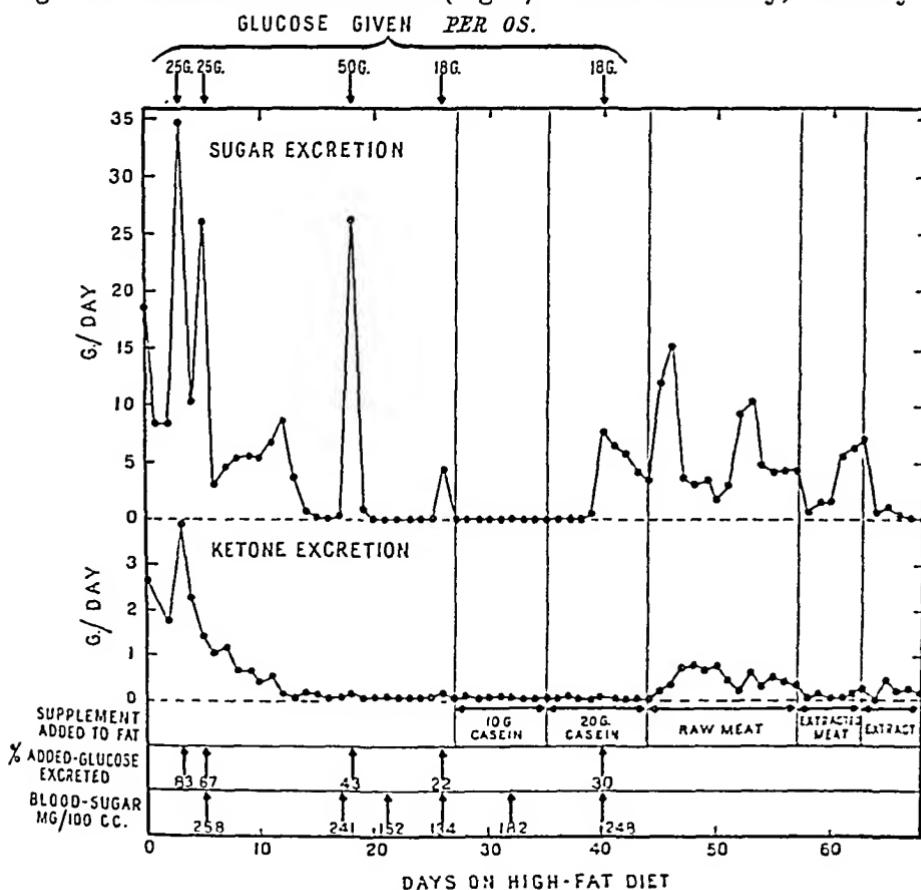
FIG. 4. Data relating to dog 44 during a period of high-fat diet.

the intravenous injection of 5 units on the 32nd day, was certainly not obviously diminished as the result of fat-feeding (Fig. 2).

As a substantial ketonuria had been observed when dog 44 was receiving a high-protein diet, it was expected that the addition of casein to the fat would result in a definite rise in ketone excretion. but this expectation was not realized [cf. Young, 1938b], as neither the daily addition of 10 g. of casein, nor the addition of double this amount, resulted in any increase in the excretion of ketones. In fact, the excretion decreased somewhat (Table XI). It should be mentioned that it was possible to estimate these small amounts of ketone with accuracy by the gravimetric method,

owing to the small volume of urine excreted by the animal during this period. 50 g. of raw horse meat were found to contain, in our experiments, about as much nitrogen as 10 g. of casein, and therefore presumably to contain somewhat less protein, since non-protein nitrogenous extractives would be present. When the supplement of casein was replaced by one of 50 g. of
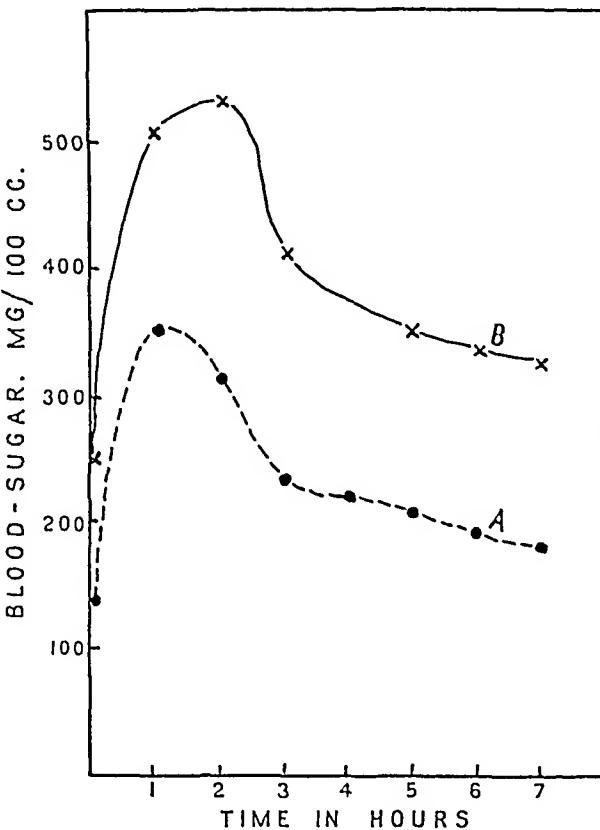
Fig. 5. Sugar-tolerance curves on dog 44 during a period of high-fat diet.

Curve A. Response to oral administration of 18 g. of glucose on the 26th day of fat feeding.
Curve B. Response to the same dose of glucose after a further 14 days, during which a daily supplement of 10–20 g. of casein was added to the diet of fat.

this raw meat, the ketonuria at once rose to an average level which was more than 25 times that observed when 20 g. of casein had been fed (Table XI). This result was surprising in view of the fact that this amount of raw meat contained only one-half the amount of protein in 20 g. of casein. Subsequently the supplement of raw meat was replaced by one of meat from which as much aqueous-soluble material as possible had been removed by repeated hot-water extraction. When a supplement of this residue was fed in amount equivalent to 100 g. of raw meat each day, the ketonuria fell to about one-fourth of the value it had reached on the supplement of

50 g. of raw meat, but when the supplement of extracted meat was replaced by a concentrate of the hot aqueous extract of raw meat, equivalent to 100 g. of raw meat each day, the ketonuria again rose, in this instance to twice the average value which had been observed during the period when the extracted meat had been fed. These observations suggest that a water-soluble constituent of raw flesh may be concerned in the aetiology of ketonuria, and that such a substance is not present in casein. It should be noted that there is no obvious relationship between glycosuria and keto-nuria to be observed from the data in Table XI. In particular it was found

Table XI. *Data relating to dog 44 during a period of high-fat diet*

Period (Initial and final day)	Average body-weight during period kg.	Supplement added daily to fat	Nitrogen content of daily sup-plement	Average glucose excre-tion g./day	Average nitrogen excre-tion g./day	D/N	Average ketone excretion g./day	Remarks
6–14	7·00	0	—	4·8	1·8	2·67	0·54	—
15–25	6·50	0	—	0·0	0·9	—	0·06	Days 18 & 19 omitted
27–35	6·25	10 g. casein	1·6 g.	0·0	1·1	—	0·06	Day 32 omitted
36–44	6·00	20 g. casein	3·2 g.	3·0	1·6	1·88	0·02	Day 40 omitted
47–57	6·00	50 g. raw meat	1·6 g.	4·7	2·7	1·74	0·53	—
58–63	5·75	Extracted residue equivalent to 100 g. raw meat	2·7 g.	3·7	2·4	1·54	0·13	—
64–68	5·75	Extract equiva-lent to 100 g. raw meat	0·45 g.	0·3	1·2	0·25	0·23	—

that, when the extract was substituted for the extracted meat, the glyco-suria decreased while the ketonuria simultaneously increased. Dog 44's daily fat intake was approximately constant during these experimental periods, at about 45 g. of beef suet each day.

A few words may be said about the significance of the results in Table XI with respect to nitrogen metabolism. From the 15th to the 25th day of fat-feeding dog 44 excreted an average of 0·9 g. of nitrogen in his urine each day, which was equivalent to 0·14 g./kg. body-weight. This figure, which may be taken as indicative of the basal nitrogen metabolism of this dog, is, as expected, significantly lower than the value of 0·23 g./kg. body-weight observed by Howe, Mattill, and Hawk [1912] for a dog which had fasted for over 16 weeks. The addition of 10 g. of casein to the daily food of dog 44 resulted in a rise of average nitrogen excretion of only 0·03 g./kg., which is hardly significant, despite the fact that 0·26 g. of nitrogen per kg. body-weight had thus been added to the food each day. When twice this amount of nitrogenous food was added in the form of casein, the nitrogen excretion rose to a figure which was still only 0·13 g./kg. above the basal level. We see, therefore, that when 10 g. of casein were added to the food—an amount of protein containing sufficient nitrogen to provide an excess of more than 80% over the basal loss in

the urine—only about 12% of the added nitrogen was lost in the urine; that is, nearly 90% was apparently retained in the body. It is clear that casein possessed a high 'biological value' in these experiments. When the amount of casein added daily was doubled, still only a small fraction of the nitrogen in this extra amount of protein was excreted in the urine.

When the daily protein supplement consisted of 50 g. of raw horse flesh, containing 0·27 g. of nitrogen per kg. body-weight, the urinary nitrogen excretion above the basal level amounted to 0·31 g./kg., so that little of the nitrogen in the raw meat appeared to be retained in this

Table XII. *Data relating to fasting diabetic dogs*
(*Mean values for 1st and 2nd days of fasting*)

Dog	Previous diet	No. of observations	Sugar excretion g./day	N excretion g./day	D/N	Ketone excretion g./day	Blood sugar g./100 c.c.	No. of observations	Sugar excretion g./day	N excretion g./day	D/N	Ketone excretion g./day	Blood-sugar g./100 c.c.
		1st Day						2nd Day					
28	Meat	1	26·5	—	—	—	0·277	1	22·8	—	—	—	0·219
41	Meat	5	23·2	7·8	2·97	1·24	0·266	2	19·5	6·9	2·82	0·11	0·286
41	'Toronto'	3	32·6	8·4	3·88	3·60	—	2	9·7	3·3	2·91	1·24	—
50	Meat	2	8·6	4·9	1·75	0·39	0·259	2	8·0	4·7	1·70	0·19	0·222
50	'Toronto'	1	9·2	4·2	2·19	4·14	—	—	—	—	—	—	—
51	Meat	4	31·6	10·6	2·98	1·20	0·401	3	12·4	4·9	2·53	0·85	0·327
60 (depancreatized)	Meat	1	28·2	10·4	2·71	2·12	—	—	—	—	—	—	—
,,	'Toronto'	2	23·2	7·1	3·25	1·85	—	—	—	—	—	—	—
Normal dogs (approx. 10 kg.)	Meat	8	0	5·9	—	0	0·081	6	0	3·9	—	0	0·083

experiment. Later, when the raw meat was replaced by thoroughly extracted meat-protein residue containing 0·47 g. of nitrogen per kg. body-weight, the nitrogen excretion above the basal level was 0·28 g./kg., so that a substantial retention of nitrogen appeared to be taking place in this instance.

Blood-sugar level

The highest blood-sugar value we have observed for any of our pituitary-diabetic dogs was just under 0·900% for dog 51. When the pituitary-diabetic animals were receiving a high-protein diet the blood-sugar varied between 0·300 and 0·450%, values more nearly approaching the latter figure usually being observed. Dog 40, which, as has already been pointed out, seemed to be somewhat less severely diabetic than the other pituitary-diabetic animals, nevertheless exhibited a well-sustained hyperglycaemia, the highest and lowest values (high-protein diet) being 0·382% and 0·328% respectively. As already noted, the blood-sugar level was high when a high-carbohydrate diet was given, but diminished when the animals were ingesting pure fat.

As will be seen from Table XII, the high blood-sugar level of the pituitary-diabetic dogs was well maintained during a short fast.

The influence of fasting on the metabolism

No experiments were carried out in which the nitrogen metabolism of our dogs was brought to a basal level by prolonged fasting. Table XII summarizes the results of fasting for 48 hours, at the end of which time there is no reason to expect that the nitrogen excretion had reached the low level it would attain on more prolonged fasting. It will be seen that on the 2nd day of fasting the D/N quotient had fallen to a figure close to the classical Minkowski value of 2·8, except in the case of dog 50, where it fell to a much lower value, even on the first day of fasting. The hyperglycaemia was well maintained during the fasting period.

Insulin-content of the pancreas of pituitary-diabetic dogs. Campbell and Best [1938] found that the insulin-content of the pancreas of one pituitary-diabetic dog was less than 2 units, and similar observations were reported by Campbell *et al.* [1939]. We found that the pancreas of pituitary-diabetic dog 44 contained a total of about 2·5 units of insulin, compared with an average value of 76 units for the pancreas of nine normal dogs of approximately the same weight.

The nature of the diabetes

If the maintenance of the diabetic state in our dogs were due to over-action of the anterior pituitary lobe, one would expect a relative insensitivity to the action of insulin of the type observed in some cases of clinical diabetes. However, no obvious insensitivity to the hypoglycaemic action of a test dose of insulin was observed, such as is found in dogs which are exhibiting a temporary state of diabetes maintained by the daily injection of anterior lobe extract [cf. Benedetto, 1933; Young, 1939 a]. De Wesselow and Griffiths [1936] observed that the plasma from certain types of patient with clinical diabetes possessed an action similar in some respects to that of anterior pituitary extract, in that the administration of such plasma to young rabbits induced, under suitable conditions, a relative insensitivity to the hypoglycaemic action of injected insulin. Although some investigators have been unable to confirm these findings, Glen and Eaton [1938] observed a very striking case of insulin-resistant clinical diabetes in which such an action of the serum was clearly demonstrable. Cogent arguments have been adduced for the assumption that such cases of diabetes are associated with overaction of the anterior pituitary lobe. We have been unable to demonstrate any effect of this type with serum from our pituitary-diabetic dogs.

Himsworth [1936] has divided human diabetic patients into two classes—insulin-sensitive and insulin-insensitive—on the basis of the results of tests in which first glucose alone and later glucose with insulin were administered to the fasting patient. When glucose with insulin was

administered to the insulin-sensitive group the hyperglycaemia was very
much less than when glucose alone was given, but with the insulin-
insensitive patients little or no depression of the glucose hyperglycaemia
resulted from the simultaneous administration of insulin. In an experi-
ment of this type with dog 44 we obtained the results illustrated in Fig. 6.
Professor Himsworth kindly examined these results for us and stated
that the curves in Fig. 6 (*b*) demonstrate a normal insulin-sensitivity, and
that those in Fig. 6 (*a*) might be expected under conditions where the
glycosuria was uncontrolled by insulin.
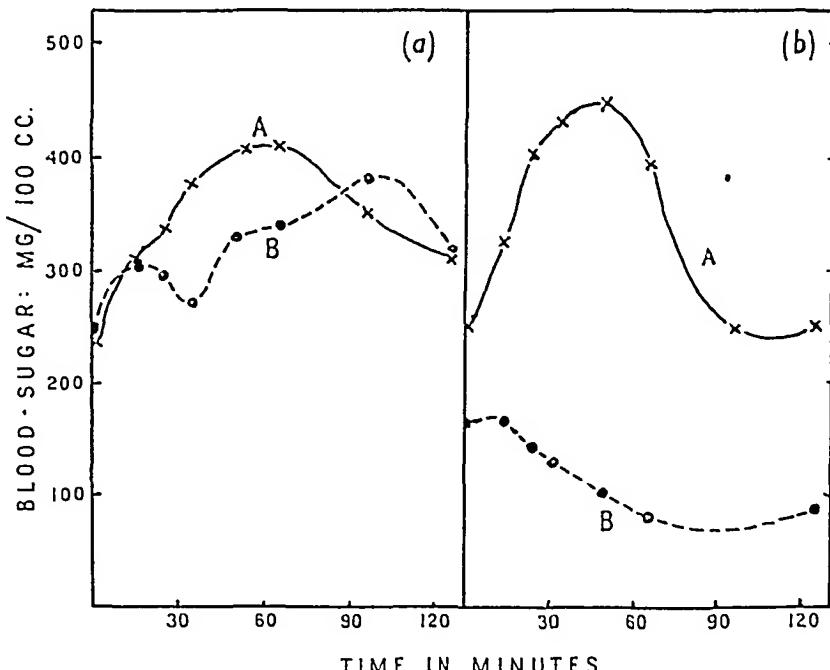
FIG. 6. Curves illustrating the results of Himsworth tests for insulin-
insensitivity on pituitary-diabetic dog 44.

(*a*) During a period when the animal was not otherwise receiving insulin.
(*b*) During a period when the glycosuria had been controlled by daily treatment with
       insulin.
Curves A. Response to 16 g. of orally administered glucose.
Curves B. Response to the oral administration of 16 g. of glucose, together with 2·5
       units of insulin injected subcutaneously.

All these results suggest that the maintenance of the diabetic condition
in our animals is not the result of continued overactivity of the anterior
pituitary gland, for a hyperpituitary-diabetes, of which we may assume
the type obtained during the daily injections of pituitary extract is an
example, would presumably be associated with a relative insensitivity
to the hypoglycaemic action of insulin. Histological examination of the
pituitary gland of permanently diabetic dogs [Richardson and Young,
1938] has revealed no obvious changes, such as might be expected to
accompany continued overactivity of this organ.

The changes in the islets of Langerhans of the pancreas may be responsible for the maintenance of the diabetic condition, but these changes are apparently not capable of explaining all the observed facts—such as the ability of these dogs to live in good condition without insulin therapy, while maintaining extremely high D/N quotients, and the excessive amount of insulin required to control glycosuria in some of these dogs.

Discussion

The results of this investigation have revealed both similarities and differences between the diabetic condition of pituitary-diabetic dogs and that of depancreatized dogs. The most striking difference is the ability of the pituitary-diabetic animals to survive, in good condition and without any great loss of body-weight, in the complete absence of insulin therapy. The intensity of the diabetes of the pituitary-diabetic animal varies from dog to dog, but ability to survive without insulin has been observed initially in all cases, although later, if the body-weight is much reduced, as by prolonged fat or carbohydrate feeding, insulin may be required to enable the animal to regain its original weight. Whether or not one of these severely diabetic dogs could survive many years without the institution of insulin therapy, if an ample mixed diet were given and enervating experimental conditions were not imposed, has not yet been determined, as all our dogs have been subjected to a variety of experimental conditions. It is clear, however, that survival of untreated pituitary-diabetic dogs cannot be expected unless ample supplies of utilizable food are given, so that the metabolic requirements of the animal are covered by the oxidation of that portion of the foodstuffs which escapes conversion into urinary glucose and ketones.

Differences of a less striking character have been observed in respect of the D/N quotients in animals maintained on a high-protein diet, and in respect of the amount of insulin required to control glycosuria. These two points will be discussed under separate headings.

The D/N quotient in protein-fed animals

As has already been pointed out, the classical D/N quotient of 2·8 considered by Minkowski [1893] to hold for completely depancreatized dogs receiving varying amounts of meat is based on a somewhat insecure foundation. However, in a very recent carefully controlled investigation, Gray, Ivy, and Cuthbert [1939] observed D/N quotients of 2·88 for fasting depancreatized dogs and 2·81 for depancreatized dogs ingesting casein. D/N quotients higher than 2·8 for completely depancreatized dogs have previously been recorded. Thus Falta, Grote, and Stæhelin [1907] observed an average value of 3·27, while Macleod [1926, 1928] and his

pupils have frequently observed values above that of Minkowski. Sandmeyer [1895], Pflüger [1905], and others have observed low values in a mildly diabetic condition following partial pancreatectomy, but Langfeldt [1920] observed quotients above 3·0 in partially depancreatized dogs in which a slowly developed but severely diabetic condition had been produced. The values above 3·0 which we have observed with both depancreatized and pituitary-diabetic dogs receiving a diet of raw meat are therefore not unique. It is interesting to note that Mandel and Lusk [1904] and others [see Lusk, 1928, for refs.] have observed a D/N quotient of 3·6 in human *diabetes mellitus*, a value which is also found in phloridzinized animals of a number of species [cf. Lusk, 1928]. Furthermore, Minkowski [1893] observed that the administration of phloridzin to depancreatized dogs may result in a rise of D/N quotient to a value similar to that found in phloridzinized but otherwise normal dogs.

Falta [Falta *et al.* 1907; Falta, 1908, 1917] has pointed out the differences existing between the condition of the human diabetic patient, with a relatively high D/N quotient and only slightly increased endogenous nitrogen catabolism, and the condition of the fully depancreatized dog with its lower D/N quotient and greatly increased protein catabolism [see also Müller, 1922; Petrén, 1923]. Our results with depancreatized dogs suggest that if the animals are maintained in good health for some time after pancreatectomy with the aid of insulin treatment, a D/N quotient above 3·0 is subsequently found when insulin is withdrawn and raw meat is fed, and that a similar, possibly slightly higher value is observed in meat-fed pituitary-diabetic dogs. It seems to us that on this score there is no obvious difference between the depancreatized dog, human diabetic patient, or pituitary-diabetic dog, when meat is the food. It is possible that the higher values for meat-fed depancreatized dogs are found particularly when the diabetic condition has existed for some time, and Petrén [1924] has emphasized the point that a pancreatic diabetes of long standing may differ in some respects from a similar but acute condition. Thus Petrén states [loc. cit., p. 52], in discussing the depancreatized dog, 'when the consequence of the insufficiency of the pancreas, i.e., the hyperglycaemia, has continued long enough, this causes the alteration of the organism that is very marked in grave diabetes, i.e., the strong sensibility to metabolized N, which I have shown to be the cause of the severe acidosis. The effect of this sensibility to N is . . . to elevate the quotient D/N.' It is certainly true that in our depancreatized dogs which were allowed to survive some weeks before being deprived of insulin, both the D/N and the ketonuria were much higher than expected from the results of Minkowski [1893] and others, whose animals survived pancreatectomy for only a short time. It should be emphasized that our depancreatized

dogs did not become fat during the period of recovery from the operation, and there is no reason to suspect that the high D/N quotients or the substantial ketonuria were due to the presence of excessive deposited fat. Again, it should be pointed out that in most of the experiments on our depancreatized dogs, food containing a large proportion of raw pancreas was given, for there is evidence that the addition of raw pancreas to the diet influences the course of glycosuria in depancreatized dogs. Thus Sandmeyer [1895] and others have observed an increase in glycosuria when raw pancreas is added to a raw-meat diet, an observation which is explicable by increased intestinal absorption, resulting from the addition of pancreatic enzymes to the food. On the other hand, Langfeldt [1920] found a higher D/N quotient when raw pancreas was added to the diet than when boiled pancreas formed the supplement, a result which is presumably not accountable merely to altered absorption from the gut. We observed no difference in D/N quotient when depancreatized dog 60 was given raw meat with or without the addition of *liquor pancreaticus* (see Table IV). There is no reason to believe that the extensive fatty infiltration of the liver observed by Macleod and his colleagues [see Macleod, 1926] to follow the prolonged maintenance with insulin of depancreatized dogs on a diet of raw meat and sugar, occurred in our depancreatized animals, as such a condition is cured or prevented by the addition of raw pancreas to the diet. Furthermore, such a condition is associated with a diminished, and not an increased, glycosuria and D/N quotient [see Best, 1934; cf. Best, Huntsman, and Young, 1935].

There is general agreement that the protein catabolism of fasting, completely depancreatized dogs is much greater than that of normal fasting dogs [cf. Lusk, 1928, for refs.], the increase being much greater than that found in human *diabetes mellitus* [Benedict and Joslin, 1912]. In his two partially depancreatized dogs, in which a chronic but severe diabetes had slowly developed, Langfeldt [1920] observed an abnormally high nitrogen excretion during fasting, although the increase above normal was much less than that found with completely depancreatized dogs. Despite the fact that the results are not certain because of the shortness of the fasting period, the figures in Table XII suggest that the breakdown of protein during short-fasting in pituitary-diabetic dogs is somewhat above that for normal dogs in our experiments, although it is clearly not so greatly increased as is generally found for depancreatized dogs. Such an increase in protein catabolism may be associated with the increase in metabolic rate (Table VI). Dohan and Lukens [1939] found that with their pituitary-diabetic dogs the nitrogen excretion during prolonged fasting was within the range of the normal animal.

All that we can state definitely about the D/N quotient of our dogs during

fasting is that a fall was observed. Whether or not such a pronounced fall as was observed by Dohan and Lukens [1939] would be found on more prolonged fasting cannot be determined.

The insulin-requirement of pituitary-diabetic dogs

The long series of experiments which have been carried out on this point has shown that the insulin-requirement of pituitary-diabetic dog 44 is nearly twice that of any of the depancreatized dogs examined. There is good reason to believe that at least two other pituitary-diabetic dogs in our series, viz. dogs 28 and 51, required as much insulin as did dog 44 for the control of glycosuria; it is unfortunate, however, that no data for the insulin-requirements under the 'Toronto' diet are available for these two animals.

During the course of these experiments many apparently inexplicable variations in the glycosuria of our dogs, pituitary-diabetic or depancreatized, were observed during periods when the conditions were apparently constant. Although some of these variations were later related to the rate at which the food was eaten by the dogs, it was obviously necessary to obtain average data from a large number of different experiments in order to be certain of the significance of the results. The difficulties inherent in the interpretation of the results are illustrated by the data regarding the effect of extirpation of the pancreas on the insulin-requirement of pituitary-diabetic dog 44 (Table VII). Although a slight diminution in insulin-requirement may actually have taken place, it seems unwise to draw a definite conclusion in view of the hardly significant change observed when only 640 g. of 'Toronto' diet were fed daily, as compared with 800 g. in the earlier experiments, although, as already pointed out, the difference in body-weight may account for the failure to observe a difference in insulin-requirement after pancreatectomy when 640 g. of food were given daily. Great caution is clearly required in comparing these results with those from other laboratories, and more particularly with those from diabetic patients. With patients, the time elapsing between insulin-administration and the taking of food, the number of doses of insulin given daily, as well as the rate of ingestion of the food, will all influence the degree to which sugar is retained, and although it would be attractive to speculate on the similarity of the pituitary-diabetic dogs to human patients with regard to the amount of glucose retained per unit of insulin, it is unwise to do so.

From our investigations there has emerged no obvious reason to account for the greater insulin-requirement of some of the pituitary-diabetic dogs as compared with that of the depancreatized animals, nor have histological observations thrown light on this matter [Richardson and Young, 1938;

Richardson, 1939–40]. Certainly the results of metabolic investigations do not preclude the possibility that the existence of the observed islet changes account for the permanently diabetic state [Young, 1939 *a*, *b*; Richardson, 1939–40; Dohan and Lukens, 1939].

The immediate and substantial hypoglycaemic response to a test dose of insulin administered subcutaneously to pituitary-diabetic dogs [Fig. 2; cf. also Dohan and Lukens, 1939] shows that these animals cannot rightfully be classed as insulin-resistant, as has been done by some commentators [cf. Dodds, 1939] presumably on the basis of the large amount of insulin required to control the glycosuria in these animals. It is not entirely paradoxical that the hypoglycaemic effectiveness of a test dose of insulin administered during fasting is apparently not abnormally low, whereas the amount of insulin which must be administered with food in order to control glycosuria may be greater than that necessary in the case of the depancreatized dog. The ability of insulin to hold down the blood-sugar level when a large amount of relatively slowly absorbed protein food is given may not be directly related to the glycaemic response of the fasting animal to insulin administered without food. The glucose-insulin test devised by Himsworth [1936] may be a closer index of the ability of insulin to control glycosuria than is a simple determination of insulin hypoglycaemia, and this test indicates that our pituitary-diabetic dog 44 is not in Himsworth's insulin-insensitive class. It should be pointed out that the reality of the division of human diabetic patients into insulin-sensitive and insulin-insensitive classes has been recently questioned [de Wesselow and Griffiths, 1938; Klatskin, 1938; Bugert, Nadler, and Stott, 1939].

The fact that pituitary-diabetic dogs are able to survive in good condition without insulin therapy may possibly be associated with a relatively small increase in endogenous nitrogen metabolism. As has been suggested [Young, 1939 *b*], the death of the depancreatized animals following the cessation of opotherapy, in conditions under which the pituitary-diabetic dog is able to survive [cf. Young, 1939 *a*], may be due to a rapid dehydration [cf. Gray *et al.*, 1939]. The pituitary-diabetic dog is able to withstand the change in conditions if the withdrawal of insulin is effected slowly, although the abrupt cessation of treatment may prove fatal even in these animals.

Oxidation of carbohydrate by pituitary-diabetic dogs

Although, on the average, the R.Q. shows no rise after the administration of a test dose of glucose (Table VI), a small proportion of the sugar is apparently retained (Table V). More particularly, when a diet containing a high proportion of preformed carbohydrate is given, the pituitary-

diabetic dog retained, and possibly oxidized, nearly 15% of the total available carbohydrate (Table IX). In spite of this, the loss of calories in the urine was so great that the animals lost body-weight rapidly. At one time many investigators would have insisted that as no oxidation of carbohydrate could take place in the complete absence of insulin, the retention of these small amounts of sugar necessarily indicated that some insulin was available in the pancreas of our pituitary-diabetic dogs. While we do not wish to assert that no insulin is there available, we wish to emphasize the fact that there is clear evidence of the ability of the completely depancreatized and hypophysectomized dog to oxidize some carbohydrate [cf. Houssay, 1936; Chambers, Sweet, and Chandler, 1937], so that the ability of our dogs to oxidize sugar would not necessarily mean that some insulin was available.

Oxidation of fat by the pituitary-diabetic dog

Our results with fat-feeding (Table X), and in particular the prolonged experiment with dog 44 (Fig. 4 and Table XI), show that our pituitary-diabetic animals are able to metabolize a diet of pure fat, in such a manner that both glycosuria and ketonuria are much less than when the diet contains a high proportion of meat. Although such a result would not be expected on the basis of the Rosenfeld-Shaffer-Woodyatt theory summed up in the aphorism 'fats burn in the flame of the carbohydrate fire' (the results of insufficient oxidation of carbohydrate being failure of oxidation of fatty acids beyond the four-carbon atom stage), it is, nevertheless, not surprising in view of the clinical evidence concerning the dietetic treatment of diabetes. As long ago as 1797 Rollo found that the inclusion of a high proportion of animal fat in the diet of diabetic patients was advantageous, and the treatment of *diabetes mellitus* by diets containing a high proportion of fat was advocated by F. M. Allen [1915, 1917; Allen, Stillman, and Fitz, 1919], by Newburgh and Marsh [1920], and by Petrén [1923, 1924]. The latter author found [1924, pp. 34 and 35] that the administration of a diet containing a very high proportion of fat rapidly diminished ketonuria; in fact, in some cases the ingestion of a diet which, according to Shaffer's calculations [1921], should be strongly ketogenic, resulted in a rapid and substantial fall in an existing ketonuria. Cases of coma were successfully treated with a high-fat diet, and in grave diabetes Petrén gave 100–200 g. of butter alone for two or three successive days. A number of instances have also been recorded of a diminution in glycosuria following the administration of fat to depancreatized [Mohr, 1906] and partially depancreatized dogs [Langfeldt, 1920], and to phloridzinized dogs [Schmid, 1905], although Page and Young [1932] found that the intravenous administration of phosphatide emulsion to phloridzinized

dogs exerted no influence on the sugar excretion. F. M. Allen [1917], who has carried out extensive investigations with partially depancreatized dogs, believes that 'fat unbalanced by adequate quantities of other foods is a poison' [loc. cit., p. 352]. Dohan and Lukens [1939] have confirmed the diminution of glycosuria and ketonuria following the administration of fat to pituitary-diabetic dogs.

The results of our investigations have shown that the ketonuria is at a maximum when a meat diet (in particular a raw-meat diet) is given, and is lowest with the prolonged feeding of fat. Thus we are led to assume that protein-food, or rather meat, is chiefly responsible for the development of ketonuria in diabetes. The prolonged experiment with dog 44 (Fig. 4 and Table XI) suggests that the promoter of ketogenesis is not the protein *per se*, but some water-soluble constituent of muscle tissue. It is of particular interest to find that Petrén [1924], on the basis of clinical investigations, also believes that protein food has a particular ketonuric effect; 'a pronounced acidosis is regularly diminished through the intake of only fat (butter). Furthermore, pure protein feeding has generally increased the degree of acidosis' [loc. cit., p. 37]. Petrén finds that the administration of a high-fat diet results in diminished glycosuria, blood-sugar level, and ketonuria, and that the feeding of protein may increase all these values, observations which are in entire agreement with the results of our own experiments, with the addition that we find that a pure protein, like casein, has little or no influence on ketonuria, whereas raw meat has a definite effect in this respect.

Investigations during the past few years have modified our views regarding the metabolism of ketone bodies. Thus Blixenkrone-Møller [1938] found that the oxidation of ketone bodies can satisfy 60–100% of the energy-requirements of the muscles of a perfused hind-limb preparation, when they are at rest or electrically stimulated. In general, the available evidence suggests that, like glucose, ketone bodies are formed in the liver and oxidized in the muscles, both in normal and in depancreatized animals [see Mirsky, Nelson, and Grayman, 1939, for references; cf. also Crandall and Ehni, 1938]. According to this idea, the amount of ketone bodies excreted in the urine represents the balance between production in the liver and utilization in the muscles. Thus there is no difficulty in explaining the diminution in ketonuria, which follows the ingestion of fat by the diabetic animal or patient, as the result of the establishment of a more accurate balance between ketone-production in the liver and ketone-utilization in the muscles. The interesting experiments of Barker [1936, 1939] are of importance in this connexion. Barker has shown that there is little or no increase in ketonuria when the metabolic rate of the fasting, depancreatized dog is increased by exercise or by dinitrophenol administration.

Moreover, the fasting depancreatized dog excretes only about 10% of the amount of ketone bodies to be expected, if one molecule of four-carbon acid is formed from each molecule of fatty acid oxidized. Thus there appears to be no obvious relationship between the extent to which fat is oxidized and the amount of ketonuria observed.

The results of our experiments, in agreement with the earlier clinical observations of Petrén [1924], suggest that the excretion of ketone bodies in diabetes is particularly associated with the oxidation of meat. Is it possible that the formation of ketone bodies is stimulated by the breakdown of body protein, or rather muscle substance, and that an increased oxidation of fatty acids does not result in an increased excretion of ketone bodies unless there is also a substantial increase in the catabolism of flesh, either intrinsic or extraneous? Such a possibility must certainly be considered in view of the presented evidence. It is unfortunate that in the past so few experiments have been made concerning the influence of administering pure fat to diabetic patients or animals. Because of the fear of precipitating coma, a good proportion of protein food such as meat was usually added to the fat, the addition of which may have completely obscured the influence of the fat. Those courageous clinicians who, like Petrén, gave food containing a high proportion of fat, but no flesh, observed a dramatic fall in ketonuria.

In assuming that the breakdown of muscle substance is largely responsible for the appearance of ketone bodies in the urine, we need not abandon the theory, based on extensive experimental evidence, that ketone bodies can be formed by the breakdown of fatty acids, although it is generally admitted that certain amino-acids are also ketogenic [Lusk, 1928]. The only necessary assumption is that in the muscles the oxidation of the ketone bodies, which are formed by the breakdown of fatty acids in the liver, is diminished when the catabolism of muscle substance is increased. There seems no justification for speculation on the possible mechanism of such a process with the evidence at present available, but any such discussion must take account of the fact stressed by Pflüger [1905] and others that 'the extent to which protein is metabolized is determined by the amount of protein ingested, whereas the extent to which fat is metabolized is quite independent of the amount of fat received in the food' [Pflüger, 1905, p. 329].

The increase in sugar tolerance which followed the administration of a pure fat diet to our pituitary-diabetic dogs appears to be at variance with the evidence that sugar tolerance is diminished by the administration of a high-fat, low-carbohydrate diet to normal animals [Abderhalden and Wertheimer, 1924] or normal men [Sweeney, 1927; Himsworth, 1935].

It seems to us that the simplest explanation of the apparent paradox is

as follows: the pancreas of the pituitary-diabetic dog contains a small amount of insulin which, however, is required little, if at all, for the regulation of metabolism when a diet of pure fat is given. This insulin therefore accumulates in the pancreas and is available to ensure the proper metabolism of at least a portion of a small dose of ingested carbohydrate, in spite of the fact that, in accordance with Himsworth's ideas, the insulin-sensitivity of the tissues may have been depressed by the high-fat diet. On such a theory we must assume that the addition of casein to a fat diet depleted the insulin-store sufficiently to diminish sugar tolerance (Fig. 5) and to cause glycosuria to reappear (Fig. 4). Whatever may be the explanation, it is clearly of interest to find that both clinically and experimentally the feeding of a high-fat diet is capable of depressing the excretion of both ketone and glucose. Certainly in our dogs the high-fat diet provided sufficient combustible material to prevent such a rapid fall of body-weight as occurred when a high-carbohydrate diet was given.

SUMMARY

1. Dogs made permanently diabetic by treatment with anterior pituitary extract differ most obviously from depancreatized dogs in the following respects:
   (a) Some of these dogs require more insulin for the control of glycosuria than do depancreatized dogs;
   (b) The pituitary-diabetic dogs are able to survive for long periods in good health without insulin therapy, if sufficient utilizable food is given. The intensity of the diabetic condition may vary from animal to animal.

2. Removal of the pancreas from a pituitary-diabetic dog resulted in a slight and possibly not significant fall in insulin requirement. The pancreas contained 2·5 units of insulin, compared with an average figure for nine normal dogs, of comparable weight, of 76 units.

3. On a protein diet, the pituitary-diabetic dogs exhibited hyperglycaemia, a substantial glycosuria and ketonuria, with a D/N quotient of over 3·0 in most instances; on a high-carbohydrate diet, these dogs retained about 15% of the total available carbohydrate in the food; on a diet of beef suet, the blood-sugar level, the glycosuria and ketonuria of these dogs were all diminished, and the sugar tolerance was increased. In one animal, which tolerated a high-fat diet for over six weeks, the addition of casein to the beef-suet diet diminished sugar-tolerance, but did not increase ketonuria, although substitution of raw meat for casein resulted in a substantial rise in ketonuria. These results support the conclusions of Petrén [1924], which were drawn from clinical investigations, that

protein (meat-food), and not fat, is particularly concerned in the aetiology of ketonuria.

4. The metabolic rate of the pituitary-diabetic dogs was somewhat above that of control normal animals under similar conditions, but the excess above normal was not so great as was found with depancreatized dogs.

5. As indicated by the hypoglycaemic effectiveness of 5 units of injected insulin, by the Himsworth [1936] glucose-insulin, test, and by the de Wesselow-Griffiths [1936] serum test, the pituitary-diabetic dogs do not possess any abnormal degree of insulin insensitivity.

It is concluded that the permanently diabetic condition of our animals may well result from the changes observed in the islets of Langerhans of the pancreas, although these changes are apparently insufficient to account for all the observed facts.

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APPENDIX

On the Determination of the Respiratory Metabolism of Dogs

By H. P. MARKS

The results of determinations of respiratory metabolism in pituitary-diabetic and depancreatized dogs have already been discussed. In the present note it is proposed to deal with some practical points arising out of the investigation.

Apparatus

A closed-circuit chamber of the Atwater-Benedict [Atwater and Benedict, 1905; Benedict and Carpenter, 1910] type was employed, and it may be noted that an apparatus of this type was also used by Macleod [1929] in studying the metabolism of depancreatized dogs.

The chamber itself consisted of a substantial metal box, of 250 litres capacity,

provided with glass windows, and with a removable lid closed by a water seal. A stout grid covered the floor of the box, which was slightly coned, so as to facilitate drainage of any urine passed during an experiment.

The carbon dioxide produced by the animal was continuously removed by pumping air from the chamber through a train of CO_2-absorption vessels, and then returning it to the chamber. For this purpose a small, three-cylinder pump was used alternately to inflate and deflate three small-sized football bladders contained in rigid glass jackets. Air was thus drawn into the jackets from the metabolism chamber, and expelled through the absorption vessels, the direction of flow being determined by flat rubber valves of the familiar type. This pump has proved capable of delivering 20 litres of air per minute over long running periods; it requires very little attention, and the rubber working parts have a long useful life, and are easily replaced.

The train of absorption vessels comprises first, a dryer, and then a CO_2 absorber, followed by a second dryer to remove the moisture given off by the soda-lime. After some experimenting, 'Protosorb'[1] was finally adopted as an absorbent for carbon dioxide, since it had a high capacity for absorbing CO_2, and did not readily form lumps impeding the free passage of gas.

In common with other brands of soda-lime, however, 'Protosorb' works best when supplied with moist gas, and its absorbing properties rapidly become impaired as it loses its own moisture to the stream of dried air passing through it. In fact, moisture is lost so rapidly that a freshly filled can will usually lose weight when first used, in spite of the fact that it is absorbing CO_2. This difficulty is overcome by using two CO_2 absorbers. When a can is partly exhausted, it is placed in the second position and a freshly filled can is placed in the first position, so that the moisture lost by the first absorbent filling is largely caught by the second filling, and so helps to revive its absorbing properties.

Containers were made from tin canisters lined with bakelized paper, so as to avoid contact of the soda-lime with the metal. The lids were effectively sealed by bedding down on plasticine, and binding with adhesive tape.

For drying the current of air, an ordinary gas washer containing concentrated sulphuric acid, followed by a bottle filled with broken pumice soaked in concentrated sulphuric acid, was found to be quite satisfactory, and could be safely used until the sulphuric acid had increased its volume by 50%.

A highly sensitive steelyard balance[2] was used for weighing the absorption vessels.

In accordance with the usual practice, fresh oxygen, stored in a counterpoised gas container over water, was admitted to the chamber as required, to replace the oxygen consumed by the animal. The volume of oxygen consumed was obtained from the fall in the level of the gas container, the cross sectional area of the container being known by measurement. The degree of purity of the oxygen was ascertained by analysis and allowed for.

Experimental procedure

At the beginning of the experiment the dog was placed in the chamber, and the pump allowed to ventilate the chamber on open circuit for 10–30 minutes

[1] Obtainable from Siebe, Gorman, Ltd.

[2] 'Decimal' balance, listed by Gallenkamp.

without being connected to the absorption vessels, and without connexion of the oxygen supply. By this means the chamber was warmed up and brought to a steady state, the contained air showing little further change in composition during the course of the experiment. The circuit was then closed through the CO_2 absorption vessels, the tap connecting the oxygen container was turned on, and the apparatus run for the experimental period, usually of two hours' duration. At the beginning and end of the experimental period the following operations were performed:

The CO_2 absorption vessels and the drying vessels following them were weighed.

Samples of air were removed from the chamber for determination of the oxygen and CO_2 content by means of the small Haldane apparatus.

The level of the oxygen container was read.

The temperature of the oxygen container, the temperature of the air within the metabolism chamber (T_1 and T_2), and the atmospheric pressure (P_1 and P_2) were taken.

Calculation of results

Any change in the temperature of the chamber during an experiment involves expansion or contraction of the contained air. If this air contracts, more oxygen is drawn in from the container than is consumed by the animal, so that the air in the chamber becomes relatively richer in oxygen, while expansion of the air in the chamber, due to rise in temperature, has the opposite effect. In calculating the volume of oxygen consumed, therefore, a correction for change in composition of the chamber air has to be added to the volume of oxygen drawn from the container, in the form: $\dfrac{V_1 O_1 - V_1 O_2}{100}$ where O_1 and O_2 are the percentage oxygen contents of the chamber air at the beginning and end of the experiment respectively, and V_1 and V_2 are the corresponding values of the effective chamber volume V, after reduction to 0° C. and 760 mm. press (N.T.P.) by means of the determined temperatures, T_1 and T_2, and pressures P_1 and P_2. The effective chamber volume is the volume of the chamber, calculated from its dimensions, plus the estimated volume of the pump and absorption vessels, less the estimated volume of the animal. The volume of oxygen drawn from the container should be similarly reduced to N.T.P. by means of the average temperature of the container, and average pressure during the experiment. Useful tables for carrying out these reductions are given by Carpenter [1921]; the tables for moist gas should be used, since the air in the chamber is practically saturated with water vapour when once a steady state has been reached, and the degree of saturation does not alter appreciably during the experiment. The volume of oxygen consumed (in litres at N.T.P.) as calculated above is conveniently expressed in litres per minute, or in calories, using a factor depending upon the respiratory quotient [Lusk, 1928, pp. 66–7].

The total increase in weight (g.) of the absorption vessels during the experiment is multiplied by 0·5038, the volume in litres at N.T.P. of 1 g. of CO_2, and then corrected for the change in CO_2 content of the chamber, in order to obtain the volume of carbon dioxide produced by the animal. The correction to be

added is $\dfrac{V_2C_2 - V_1C_1}{100}$, where C_1 and C_2 represent the percentages of carbon dioxide in the air of the chamber at the beginning and end of the experiment.

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